## REMARKS

Claims 43-66 are pending in the instant application. Non-elected claims 1-24 and 39-42 have been canceled without prejudice to the prosecution thereof in a subsequent application. Claim 43 was amended to pursue preferred embodiments; claims 44 and 45 were canceled without prejudice to the prosecution of the subject matter thereof in a subsequent application. Claims 46 and 47 were amended to pursue preferred embodiments. Claim 51 was amended for clarity. Claim 61, 62, 64and 65 were amended to pursue preferred embodiments. Dependent claims 67-69 were added. The amendments and added claims are supported in the specification, particularly in the Examples, original claims, and throughout the specification (e.g., see page 13, lines 18-30) original claim 37; Example 7, page 50-52; Example 13 page 59-61, particularly page 60, lines 3-5, and lines 30-31). The instant claims are drawn to soluble zcytor11-comprising polypeptides and methods of use therefor. No new matter was added by these amendments.

*A. Rejections Addressed from March 4, 2004 Office Action (OA)*

*(1) Rejection of claims 61-66 under 35 U.S.C. § 102(a) and (e)*

Claims 61-66 were rejected under 35 U.S.C. §102(a) and (e) as being anticipated by Lok, et al., (US Patent No. 5,965,704, October 12, 1999, filed August 5, 1997). The Office states that claims 61-66 were rejected under 35 U.S.C. § 102(a) and (e) because "The '704 patent teaches a protein of residues 18-228 of SEQ ID NO:2, that is identical to SEQ ID NO:3...Fc fusions and other modifications...The properties of forming a monomeric or heterodimeric receptor, and the ability to bind IL-TIF, are inherent...." (OA, p. 3) Applicant respectfully traverses this rejection as applied to the instant claims 61-66, and as may apply to newly added claims 67-69.

Under 35 U.S.C. §102(a) or (e), for a prior reference to anticipate, every element of the claim must be included in a single reference. Not every element of the claim is included in the Lok et al. reference; therefore, it does not anticipate the claims. Moreover, for a limitation to be considered "inherent," the single reference "must describe and enable the invention, including all the claim limitations, with sufficient clarity and detail to establish that the subject matter

already existed in the prior art, and that its existence was recognized by persons of ordinary skill in the field of the invention ... *An inherent limitation is one that is necessarily present; invalidation based on inherency is not established by 'probabilities of possibilities.'*" (*Elan Pharmaceuticals Inc. v. Athena Neurosciences, Inc.* 304 F.3d 1221, 1228 (Fed Cir. 2002); emphasis added) The Lok et al. reference does not teach "with sufficient clarity and detail to establish that the subject matter" i.e., zcytor11 receptor homodimers, heterodimers and multimers, "already existed in the prior art, " and in fact teaches nothing about zcytor11 homodimers, heterodimers and multimers nor ligands that may bind the zcytor11 receptor. In fact the art of the zcytor11 receptor has demonstrated that zcytor11 can associate with different specific heterologous receptor subunits that bind different ligands. For example, the instant specification demonstrates that zcyto18 (IL-TIF; IL-22) binds to a receptor comprising zcytor11 (IL-22RA) and CRF2-4 (IL-10RB), but it has also been determined that IL-20, as well as IL-24 can bind to a receptor comprising zcytor11 (IL-22RA) and pDIRS1 (IL-20RB). For the convenience of the Office, the following references are provided to demonstrate that zcytor11 associates with multiple subunits, and different ligands other than IL-TIF: (1) Dumoutier, L. et al., J. Immunology 167:3545-3549, 2001 (copy enclosed) (See, abstract, lines 9-11, and supporting text); and (2) Parrish-Novak et al, J. Biochem. 277:47517-47523, 2002 (Copy enclosed) (See, abstract, lines 8-9, and supporting text). Consequently, it not could not have been necessarily present when Lok et al was filed, that zcytor11, nor the zvytor11-comprising receptor complexes of the present invention would (1) associate with any specific Class I or Class II cytokine receptor subunit, e.g., the CRF2-4 subunit, nor (2) that zcytor11 would inherently bind or antagonize IL-TIF.

Insofar as the binding of any ligand to zcytor11, the Lok reference merely teaches half of the equation (zcytor11) and nothing more. Given Lok, one of skill in the art could not have predicted that zcytor11 (IL-22RA) would bind or antagonize IL-TIF, and would be capable of signaling in the presence of IL-TIF; nor does the reference teach that zcytor11 could comprise a heterodimeric or multimeric receptor with CRF2-4. The claim limitations of binding or antagonizing IL-TIF, and zcytor11 comprising homodimeric, heterodimeric or multimeric receptors are not ones that are necessarily present, and therefore invalidation based on inherency

is not established by the reference. Because the reference does not teach zcytor11 receptor homodimers, heterodimers or multimers, nor the activity of binding or antagonizing IL-TIF, it does not teach every element of the claims. Nor does the reference teach or suggest that The Zcytor11 cytokine receptor, by its nature forms a homodimeric, heterodimeric or multimeric receptor complex. Therefore the reference cannot anticipate the claimed invention. Consequently, the rejection of instant claims 61-66, and as may apply to newly added claims 67-69, under 35 U.S.C. §102(a) and (e) should be properly withdrawn

*(2) Rejection of claims 61-66 under 35 U.S.C. § 102(b)*

Claims 61-66 were rejected under 35 U.S.C. §102(b) as being anticipated by Lok, et al., (WIPO Publication 99/07848, published July 30, 1998). The Office states that claims 61-66 were rejected under 35 U.S.C. § 102(a) and (e) because "The [publication] also teaches a protein of residues 18-228 of SEQ ID NO:2, that is identical to SEQ ID NO:3...[and] fusion proteins" (OA, p. 3) Applicant respectfully traverses this rejection as applied to the instant claims 61-66, and as may apply to newly added claims 67-69.

WIPO Publication 99/07848, published July 30, 1998, is identical in disclosure to by Lok, et al., (US Patent No. 5,965,704, October 12, 1999, filed August 5, 1997) cited in the 35 U.S.C. §102(a) and (e) rejection in Part A(1) above.

Under 35 U.S.C. §102(b), for a prior reference to anticipate, every element of the claim must be included in a single reference. Not every element of the claim is included in the Lok et al. reference; therefore, it does not anticipate the claims. Consequently, as detailed in Part A(1) above, the reference does not teach zcytor11 receptor homodimers, heterodimersor multimers (e.g., comprising CRF2-4), nor the activity of binding or antagonizing IL-TIF, it does not teach every element of the claims. Nor does the reference teach or suggest that The Zcytor11 cytokine receptor, by its nature forms a homodimeric, heterodimeric or multimeric receptor complex. Therefore the reference cannot anticipate the claimed invention. Consequently, the rejection of instant claims 61-66, and as may apply to newly added claims 67-69, under 35 U.S.C. §102(b) should be properly withdrawn

*(3) Rejection of claims 51-53 under 35 U.S.C. § 112, first paragraph (Written description)*

Claims 51-53 were rejected under 35 U.S.C. §112, first paragraph, for failing to comply with the written description requirement. The Office states "This is a new matter rejection. There is no mention of serum amyloid A protein in the specification as filed." (OA, p. 4) Applicant respectfully traverses this rejection.

The instant claim 51 was amended to include the commonly known acronym for serum amyloid A, "SAA," which is disclosed in Example 13 (p.59-60) within the specification. No new matter was added by this amendment. SAA is an acute phase response protein that is increased in the presence of zcyto18 (commonly known as IL-22): "The SAA and globulin level was increased and glucose level was decreased. ... In summary, zcyto18 causes acute phase response (APR) that reflects the pro-inflammatory activity of TNF-□, IL-1, and gp130 cytokines." (p. 60, lines 9-12). Moreover, for the convenience of the Office, the following references are provided to demonstrate that it was well known in the art at the time the application was filed that "SAA" was the acronym for "serum amyloid A": (1) Dumoutier, L. et al., Proc. Nat'l. Acad. Sci. 97:10144-10149, 2000 (copy enclosed) (Abbreviations, page 10144, column 2 footnote, line 2: "SAA, serum amyloid A"; and "IL-TIF induces acute phase reactant production" and "Strongly induced the expression of SAA", page 10146, column 2, lines 1-10 of the last paragraph); and (2) Uhlar, CM and Whitehead, AS, Eur. J. Biochem. 265:501-523, 1999, abstract and review article on the "serum amyloid A (SAA) family".

It is well understood in Patent Law that "information contained in any one of the specification, claims, or drawings of the application as filed may be added to any other part of the application without introducing new matter." MPEP 2163.06. Applicant has described the serum amyloid A as SAA in the specification (p. 60, lines 9-12). Consequently, as no new matter has been introduced into claims 51-53, and "SAA" was described verbatim within the specification, the rejection under 35 U.S.C. §112, first paragraph, for failing to comply with the written description requirement should be properly withdrawn.

*(4) Rejection of claims 43-45 under 35 U.S.C. § 112, first paragraph (Enablement)*

Claims 43-45 were rejected under 35 U.S.C. §112, first paragraph, for failing to comply with the enablement requirement. The Office states "the claim(s) contains subject matter which is not described...in such a way as to enable one skilled in the art...to make and/or use the invention" (OA, p. 4). Claims 44 and 45 were canceled; consequently this rejection is moot as applied thereto. Applicant respectfully traverses this rejection as applied to the instant claims 43, and dependent claim 48.

In making the rejection the Office contends, "These claims are drawn to methods of inhibiting IL-TIF-Induced proliferation of hematopoeitic cells. The specification teaches on page 60, lines 5-7 that IL-TIF is inhibitory rather than proliferative. Thus a skilled artisan would not exect inhibiting its action to have the desired result. Thus without further guidance, it would require undue experimentation...to practice the invention." (OA, p. 4)

The instant claims are drawn to methods of using the zcytor11-comprsing soluble receptors of the present invention to inhibit IL-TIF-induced proliferation of neutrophils or platelets. Applicant has clearly demonstrated that IL-TIF increases, and hence induces, the level of neutrophils and platelets *in vivo* (see Example 13, particularly lines 4-5 and 30-31). Moreover, making the zcytor11-comprsing soluble receptors of the present invention, and using the zcytor11-comprsing soluble receptors of the present invention inhibit IL-TIF activity is well documented throughout the specification. Hence, one of skill in the art would know how to make and use the zcytor11-comprsing soluble receptors of the present invention to inhibit IL-TIF-induced proliferation of neutrophils or platelets. Nothing more is needed to practice the invention, and hence there would be no undue experimentation.

The instant specification provides sufficient disclosure and guidance for one of skill in the art to make and use the polypeptides of the present invention without undue experimentation, which is all the enablement requirement of 35 USC §112, first paragraph, requires. Consequently, Applicant respectfully requests that, the rejection of claims 43 and dependent claim 48, be properly withdrawn.

*(5) Rejection of claims 46-60 under 35 U.S.C. § 112, first paragraph (Enablement)*

Claims 46-60 were rejected under 35 U.S.C. §112, first paragraph, for failing to comply with the enablement requirement. The Office states "the claim(s) contains subject matter which is not described...in such a way as to enable one skilled in the art...to make and/or use the invention" (OA, p. 4). Applicant respectfully traverses this rejection as applied to the instant claims 46-60.

In making the rejection the Office contends, "These claims are drawn to methods of treating inflammatory conditions or suppressing immune responses by inhibiting IL-TIF. The specification teaches that IL-TIF binds to zcytor11 and has pro-inflammatory effects (p. 60, lines 13-15)... there are no teachings as to when IL-TIF is produced, and thus when administration of soluble zcytor11 would be beneficial. ." (OA, p. 5)

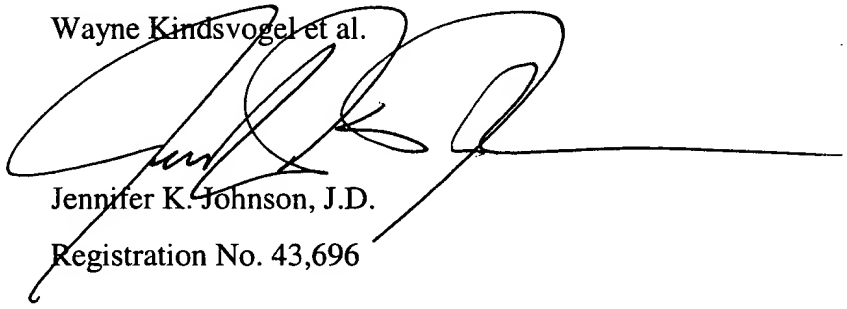
Applicant has clearly demonstrated that IL-TIF induces inflammation, and is a pro-inflammatory molecule (Example 13, e.g., page 60 lines 9-18) that enhances inflammation and inflammatory response *in vivo*. Moreover, making the zcytor11-comprsing soluble receptors of the present invention, and using the zcytor11-comprsing soluble receptors of the present invention to suppress or reduce IL-TIF pro-inflammatory activity is well documented throughout the specification. Moreover, Applicant has clearly identified diseases which would provide benefit of such suppression or reduction of IL-TIF pro-inflammatory (e.g., page 13, lines 20-30). Hence, one of skill in the art would know how to make and use the zcytor11-comprsing soluble receptors of the present invention to inhibit IL-TIF to reduce inflammation or suppress an inflammatory response. Nothing more is needed to practice the invention, and hence there would be no undue experimentation.

The instant specification provides sufficient disclosure and guidance for one of skill in the art to make and use the polypeptides of the present invention without undue experimentation, which is all the enablement requirement of 35 USC §112, first paragraph, requires. Consequently, Applicant respectfully requests that, the rejection of claims 46-60 be properly withdrawn.

Early reconsideration and allowance of the pending claims is respectfully requested. If the Patent Examiner believes that a telephone interview would expedite prosecution of this patent application, please call the undersigned at (206) 442-6676.

Respectfully Submitted,

Wayne Kindsvogel et al.



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Registration No. 43,696

Enclosures:

Amendment Fee Transmittal (in duplicate)

Petition and Fee for 3 Month Extension of Time (in duplicate)

Copies of 4 references

Postcard

# Human interleukin-10-related T cell-derived inducible factor: Molecular cloning and functional characterization as an hepatocyte-stimulating factor

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**IL-10-related T cell-derived inducible factor (IL-TIF or IL-21) is a new cytokine structurally related to IL-10 and originally identified in the mouse as a gene induced by IL-9 in T cells and mast cells. Here, we report the cloning of the human IL-TIF cDNA, which shares 79% amino acid identity with mouse IL-TIF and 25% identity with human IL-10. Recombinant human IL-TIF was found to activate signal transducer and activator of transcription factors-1 and -3 in several hepatoma cell lines. IL-TIF stimulation of HepG2 human hepatoma cells up-regulated the production of acute phase reactants such as serum amyloid A, α1-antichymotrypsin, and haptoglobin. Although IL-10 and IL-TIF have distinct activities, antibodies directed against the β chain of the IL-10 receptor blocked the induction of acute phase reactants by IL-TIF, indicating that this chain is a common component of the IL-10 and IL-TIF receptors. Similar acute phase reactant induction was observed in mouse liver upon IL-TIF injection, and IL-TIF expression was found to be rapidly increased after lipopolysaccharide (LPS) injection, suggesting that this cytokine contributes to the inflammatory response *in vivo*.**

IL-10-related T cell-derived inducible factor (IL-TIF) is a new cytokine that was originally identified as a gene induced by IL-9 in murine T lymphocytes and showed a weak but significant amino acid identity with IL-10 (1). Mouse IL-TIF consists of 179 amino acids, including four cysteins and shows 22% sequence identity with IL-10. However, IL-10 and IL-TIF do not share the same receptor complex, as the latter failed to activate signal transducer and activator of transcription-3 (STAT-3) in mouse macrophages or to induce the proliferation of IL-10-responsive MC9 cells. This cytokine is expressed by ConA-activated spleen cells and by T helper cells and mast cells, upon activation by IL-9. A weak constitutive expression also has been detected in thymus and brain (1). The biological activities of IL-TIF remain elusive. In rodents, two IL-TIF-responsive cell lines have been identified. IL-TIF activates STAT factors in PC12, a rat pheochromocytoma cell line often used as a model for neuronal cells, pointing to a putative role for this new cytokine in the nervous system. A similar effect has been observed on Mes13, a kidney mesangial cell line, but not on a series of lymphoid and macrophage cell lines (1), suggesting that IL-TIF acts mainly outside the immune system.

As a further step in our analysis of this new cytokine, we attempted to identify its human counterpart. Here, we describe the cloning of the cDNA encoding the human homolog of mouse IL-TIF. By using recombinant IL-TIF, we showed that this cytokine induces acute phase reactant expression by hepatocytes, suggesting a role for this factor in inflammatory processes.

## Materials and Methods

**Cell Cultures, Transfections, and Cytokines.** BW5147 murine lymphoma cells were grown in Iscove-Dulbecco's medium supplemented with 10% fetal calf serum (FCS), 50 μM 2-ME, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine. Human embryonic kidney 293 cells-Epstein–Barr virus nuclear antigen (HEK293-EBNA) were grown in DMEM medium supplemented with 10% FCS. HepG2 human hepatoma cells were grown in DMEM medium supplemented with 10% FCS, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine.

Recombinant mouse IL-9 was produced in the baculovirus system and purified as previously described (2). For transient expression, the IL-TIF cDNA was cloned into pCEP-4 plasmid (Invitrogen, Groningen, The Netherlands) under the control of the cytomegalovirus promoter. HEK293-EBNA cells were seeded in 6-well plates (Nunc, Roskilde, Denmark) at three $10^5$ cells/well 1 day before transfection. Transfections were carried out by using the Lipofectamine method (Life Technologies, Gent, Belgium), according to the manufacturer's recommendations with 2 μg of plasmid DNA. After transfection, cells were incubated in 1.5 ml of normal medium for 3 days for maximal production of recombinant human IL-TIF. Human IL-6 (five $10^8$ units/mg) produced in *Escherichia coli* was kindly provided by W. Sebald (Institut für Physiologische Chemie der Universität Würzburg, Germany). Anti-gp130 and anti-hIL-10Rβ antibodies were purchased from R & D Systems.

**hIL-TIF cDNA Cloning.** Human peripheral blood mononuclear cells were cultured for 24 h either with or without anti-CD3 mAb (OKT3, ascites fluid 0.2%). Total RNA was isolated by using guanidinium isothiocyanate lysis and CsCl gradient centrifugation (3). Reverse transcription (RT) was performed on 5 μg of total RNA with an oligo(dT) primer. cDNA corresponding to 50 ng of total RNA was amplified for 25 cycles by PCR with specific primers for murine IL-TIF as follows: sense 5′-AGCTGCTCAACTTCACCCTG-3′ and antisense 5′-CAAGTCTACCTCTGGTCTCAT-3′, with an annealing temperature of 45°C. The sequence of the PCR product was obtained with an automated fluorescence-based system (Applied Biosystems 310)

Abbreviations: IL-TIF, IL-10-related T cell-derived inducible factor; CHX, cycloheximide; GRR, γ response region; SAA, serum amyloid A; STAT, signal transducer and activator of transcription; mIL, mouse IL; LPS, lipopolysaccharide; RT, reverse transcription; TK, thymidine kinase; HEK293-EBNA, human embryonic kidney 293 cells-Epstein–Barr virus nuclear antigen; Tm, melting temperature.

Data deposition: The sequence of the human IL-TIF cDNA reported in this paper has been submitted to the European Molecular Biology Laboratory (EMBL)/GenBank database (accession no. AJ277247).

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using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems, Foster City, CA).

The 5′-end of the hIL-TIF message was isolated by using the 5′-RACE kit (Life Technologies) according to the manufacturer's recommendations. In brief, first strand cDNA was prepared by using a primer specific to the hIL-TIF sequence: 5′-TGGCCAGGAAGGGCACCACCT-3′. The cDNA was tailed using terminal transferase and dCTP. cDNA was amplified for 35 cycles by using an internal oligonucleotide specific for hIL-TIF 5′-TGGCCAGGAAGGGCACCACCT-3′ and the 5′-RACE-abridged anchor primer from the kit. Seminested amplification was performed on 5 μl of PCR product (diluted 1/100) using the same hIL-TIF primer and the abridged universal amplification primer from the 5′-RACE kit. Amplification was performed for 30 cycles, with an annealing temperature of 56°C, and using *Taq* polymerase from Takara (Takara Shuzo, Shiga, Japan). The resulting PCR product was cloned and sequenced. Amplification, cloning, and sequencing were repeated to avoid PCR-induced errors in the sequence.

**STAT Activation and Luciferase Assays.** Nuclear extracts were prepared, and analysis of DNA binding activity was performed as described previously (4) by using $^{32}$P-labeled oligonucleotide probes corresponding to the γ response region (GRR) of the Fc γ RI gene: upper strand, 5′-ATGTATTTCCCAGAAA-3′ and lower strand, 5′-CCTTTTCTGGGAAATAC-3′. Supershifts were performed by adding antibodies to the incubating mixture of nuclear extracts and labeled DNA probe. A 0.75 μg of anti-STAT-1 antibody (catalog no. G16920, Transduction Laboratories, Lexington, KY), 1 μg of anti-STAT-3 antibody (clone ST3–5G7, Zymed, San Francisco, CA), or 1 μg of anti-STAT-5b antibody (835-X, Santa Cruz) were used per lane.

The reporter pGRR5 was kindly provided by P. Brennan (Imperial Cancer Research Fund, London, UK). This construct contains five copies of the GRR sequence inserted upstream a luciferase gene controlled by the thymidine kinase (TK) promoter. As an internal control, we used the pRL-TK vector (Promega) containing the *renilla* luciferase gene under the control of the TK promoter. The $10^6$ HepG2 cells were transfected with 15 μg of pGRR5 and with 1 μg pRL-TK (250 V, 74 Ω, 1,200 μF). The pool of transfected cells was divided in 24-well plates (42,000 cells/well) and one part of the cells was preincubated with anti-IL-10Rβ antibodies (20 μg/ml) whereas the other part was left unstimulated. After 1 h, cells were stimulated either with hIL-TIF (1% HEK293 cell supernatant), with hIL-6 (300 units/ml) or control medium (1% supernatant from mock-transfected HEK293 cells or medium alone). Two hours later, cells were pelleted and lysed. Luciferase activity was monitored with the Dual-Luciferase Reporter Assay System kit (Promega).

**RT-PCR Analysis of Acute Phase Protein.** Five $10^6$ HepG2 cells were stimulated for 2, 13, or 24 h with 1% of supernatants from transiently transfected HEK293-EBNA cells. Protein synthesis inhibitor cycloheximide (Sigma) was used at 10 μg/ml. Total RNA was isolated using the TRIzol reagent, according to the manufacturer's recommendations (Life Technologies). RT was performed on 10 μg of total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng of total RNA was amplified for 18 cycles with primers specific for human serum amyloid A (SAA) as follows: sense 5′-AGCTCAGCTACAGCACAGAT-3′ and antisense 5′-CCTGCCCCATTTATTGGCAG-3′ [melting temperature (Tm): 54°C]; for human α1-antichymotrypsin, sense 5′-TGTCCTCTGCCACCCTAACA-3′ and antisense 5′-TAATTCACCAGGACCATCAT-3′ (Tm: 52°C); for human haptoglobin, sense 5′-GTGGACTCAGGCAATGATGT-3′ and antisense 5′-ACATAGAGTGTTAAAGTGGG-3′ (Tm: 52°C); and for human β-actin, sense 5′-GCTGGAAGGTGGACAGCGAG-3′ and antisense 5′-TGGCATCGTGATGGACTCCG-3′ (Tm: 56°C). The postPCR products were analyzed in ethidium bromide-stained agarose gel.

**Expression in *E. coli* of Mouse IL (mIL)-TIFα.** The mIL-TIFα sequence (corresponding to amino acids Q29 -V179) was amplified by PCR from the cDNA clone using primers TIFN (5′-GCCCTGTGGGCCCATATGCAGGAGGCAAATGCG-3′) and TIFb (5′-TCTTCTCGCTCAGGATCCTTAGACGCAAGCATTTCTC-3′). The PCR product was digested with *Nde*I and *Bam*HI and cloned into the pET3A plasmid (Stratagene, La Jolla, CA). *E. coli* strain BL21-codon plus-(DE3)-RIL (Stratagene) was used as the expression host. The cells were grown in Luria–Bertani medium supplemented with ampicillin (100 μg/ml), chloramphenicol (34 μg/ml), and glucose 2% (wt/vol). Expression of IL-TIF was induced with 1 mM isopropyl-β-D-thiogalactoside at a cell density (600 nm) of ≈1.3. Cells were collected by centrifugation 4 h after induction. The cell pellet was disrupted with a high-pressure cell homogenizer, and the IL-TIF inclusion bodies were collected by centrifugation. Inclusion bodies were washed extensively first with Tris·HCl 50 mM, NaCl 100 mM, EDTA 1 mM, DTT 1 mM, and sodium deoxycholate 0.5% (wt/vol), pH 8, and finally with the same buffer without detergent.

Inclusion bodies were solubilized overnight at 4°C in 8 M urea, Mes 50 mM, EDTA 10 mM, and DTT 0.1 mM, pH 6.5. The solution was centrifuged for 1 h at 100,000 × *g* and the supernatant stored at −80°C until use. The purity of the IL-TIF was estimated at ≈90% based on SDS-PAGE and Coomassie blue staining analysis. The concentration of protein was estimated by UV absorbance in urea solution by using a calculated molar absorption coefficient $\varepsilon_{280}$ = 3,840 liter/mol × cm.

The IL-TIF protein was refolded by direct dilution of the solubilized inclusion bodies in the following folding mixture: IL-TIF 100 μg/ml, Tris·HCl 100 mM, EDTA 2 mM, L-arginine 0.5 M, reduced glutathion 1 mM, and oxidized glutathion 0.1 mM, pH 8. The solution was incubated for 20 h at 4°C. The folding mixture was then concentrated by ultrafiltration in an Amicon chamber with a YM3 membrane before purification on a Superdex75 (Amersham Pharmacia Biotech) gel filtration column. The protein was eluted with Tris·HCl 20 mM and NaCl 50 mM, pH 7.

***In Vivo* Induction of Murine SAA and IL-TIF Expression.** Various amounts of recombinant mIL-TIF (50, 12.5, 3.2, 0.8, and 0.2 μg) were injected i.p. into endotoxin-resistant C3H/HeJ female mice (10–12 weeks old). Mice were killed 6 h later. Mice receiving 50 μg of recombinant mIL-TIF were killed after 1, 3, 6, 12, or 24 h. The liver was directly frozen in liquid nitrogen. Total RNA was isolated by using the TRIzol reagent, according to the manufacturer's recommendations (Life Technologies). Total RNA (10 μg) was fractionated in a 1.3% agarose gel containing 2.2 mol/liter formaldehyde before transfer onto a Hybond-C Extra nitrocellulose membrane (Amersham Pharmacia Biotech). The murine SAA probe was labeled by using the Rediprime DNA labeling kit from Amersham. Hybridization and washes were performed as described (5). The murine SAA probe was obtained by PCR with specific oligonucleotides as follows: sense 5′-TCTGCTCCCTGCTCCTGGGA-3′ and antisense 5′-TCCAGGAGGTCTGTAGTAAT-3′. The PCR was performed on cDNA synthesized from liver RNA isolated as described above and the PCR product was cloned.

For IL-TIF induction, 2 μg of *E. coli* LPS (Sigma, Bornem, Belgium) were injected i.p. into BALB/c female mice (12 weeks old). Mice were killed 2 h later and their organs were frozen in liquid nitrogen. Total RNA was isolated by using the TRIzol reagent (Life Technologies). RT was performed on 10 μg of total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng of total RNA was amplified for 27–35 cycles with primers specific

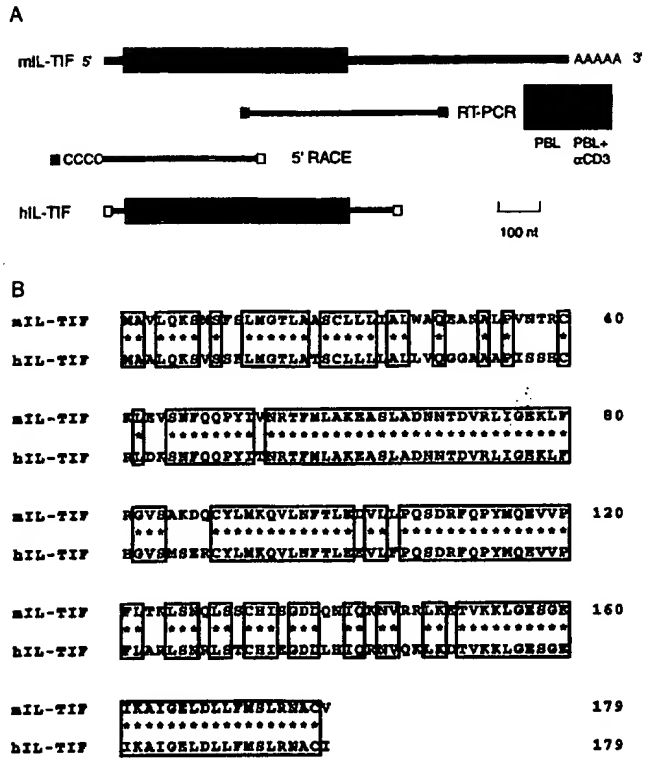


Fig. 1. Human IL-TIF cDNA cloning and protein sequence. (*A*) Total RNA was extracted from human peripheral blood mononuclear cells cultured for 24 h with or without anti-CD3 mAb. RT-PCR was performed with oligonucleotides specific for mIL-TIF (■). (*Upper*) The mIL-TIF mRNA is represented with the ORF as a black box. The 5'-end was amplified by a 5'-RACE experiment with an oligo specific to human IL-TIF (□) and with a second oligo specific to the dC tail. Finally, the full-length ORF of the hIL-TIF cDNA was amplified using specific primers (□). (*B*) Alignment of murine IL-TIF and human IL-TIF protein sequences: conserved residues are boxed.

for mIL-TIF as follows: sense 5'-CTGCCTGCTTCTCATTGCCCT-3' and antisense 5'-CAAGTCTACCTCTGGTCTCAT-3' (Tm: 55°C). The PCR products were analyzed by agarose gel electrophoresis.

## Results

**Characterization of the Human IL-TIF cDNA.** Because the IL-TIF mRNA was strongly expressed by ConA-activated mouse T-cells, we used anti-CD3-stimulated peripheral blood mononuclear cells as a source for its human homolog. A series of oligonucleotide primers were designed from the murine IL-TIF sequence and used in RT-PCR reactions. As shown in Fig. 1*A*, by using one primer in the middle of the mouse ORF and another primer from the 3'-untranslated region, we were able to amplify a message of 454 nucleotides that was present in anti-CD3-activated, but not in resting peripheral blood mononuclear cells. This fragment was sequenced and the 5'-end of the cDNA was isolated by the 5'-RACE method. Finally, oligonucleotides that flanked the coding sequence allowed for the amplification of a 690-nucleotide fragment, including a 537-bp ORF that encodes a 179-aa protein. Human IL-TIF has the same length and shares 79% amino acid identity with mIL-TIF (Fig. 1*B*) and 25% identity with human IL-10. Further analysis of the GenBank databases showed that the last exon of IL-TIF is present on a bacterial artificial chromosome clone derived from chromosome 12q15 (accession no. AC007458; 191,111 bp; bacterial artificial chromosome RPCI11–444B24). Based on this bacterial artificial chromosome sequence, the IL-TIF gene is located at ≈90 kb of the IFNγ gene and at <30 kb of the AK155 gene, another IL-10-related cytokine gene that was described recently (6).

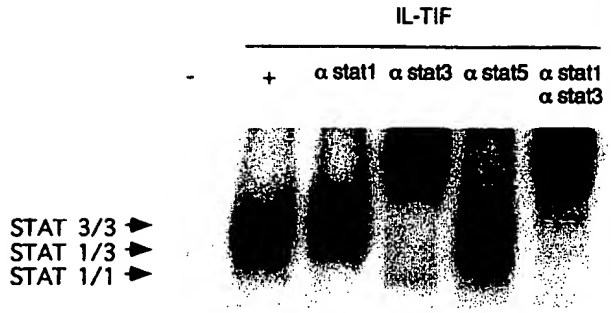


Fig. 2. STAT activation by IL-TIF in HepG2 cells. HepG2 cells were stimulated for 15 min with hIL-TIF (1% 293-EBNA cell supernatant) or with a supernatant from mock-transfected cells. Gel shift assays were performed as described in *Materials and Methods*, with the FcγRI-derived GRR probe. STAT-containing complexes were supershifted with anti-STAT-1, -3, or -5 antibodies as indicated.

**Identification of hIL-TIF-Responsive Cell Lines.** Recombinant human IL-TIF protein was produced by transient transfection of HEK293 cells. Such HEK293 cell supernatants were used to search for new IL-TIF responsive cell based on the hypothesis that hIL-TIF, like mIL-TIF, would induce activation of STAT transcription factors in target cells. Using an electrophoretic mobility shift assay with the FcγRI-derived GRR sequence, which binds to all STAT factors, we screened a number of cell lines for the nuclear translocation of STAT transcription factors after 15 min of hIL-TIF stimulation.

In this assay, an IL-TIF-induced bandshift was observed with the hepatoma cell line, HepG2. Further characterization of STAT factors that are activated in response to IL-TIF in HepG2 cells was achieved by supershift experiments with antibodies specific for STAT-1, -3, and -5. As shown in Fig. 2, anti-STAT-3 antibodies supershifted most of the retardation complexes, and the weak remaining complexes were supershifted by anti-STAT-1, whereas anti-STAT-5 antibodies had no effect. Thus, STAT-3, and to a lesser extent STAT-1, are the major STAT transcription factors activated by IL-TIF in this hepatoma cell line. Similar results were obtained with another human hepatoma cell line, HepG3, and with the rat hepatoma, H4IIE, using both human and mouse IL-TIF. As previously observed with mouse cell lines, IL-TIF failed to activate STAT transcription factors in lymphoid cell lines such as Epstein–Barr virus-transformed B cells. By contrast, four of nine melanoma cell lines showed STAT-3 activation in response to IL-TIF, indicating that the activity of this new cytokine is not restricted to hepatocytes (data not shown).

**IL-TIF Induces Acute Phase Reactant Production.** Because activation of STAT-3 by cytokines such as IL-6 is known to result in acute phase protein induction in hepatoma cells (7, 8), we tested the possibility that IL-TIF exerts the same activity. HepG2 cells were stimulated with and without IL-TIF, and RT-PCR was used to assess the expression of SAA, α1-antichymotrypsin, and haptoglobin. As shown in Fig. 3, IL-TIF strongly induced the expression of SAA, α-1 antichymotrypsin, and, to a lesser extent, haptoglobin. By contrast, IL-10 was not active in this assay (data not shown). To determine whether IL-TIF directly up-regulates SAA expression or whether this process requires protein synthesis, HepG2 cells were stimulated with hIL-TIF in the presence of cycloheximide. As shown in Fig. 4, cycloheximide did not

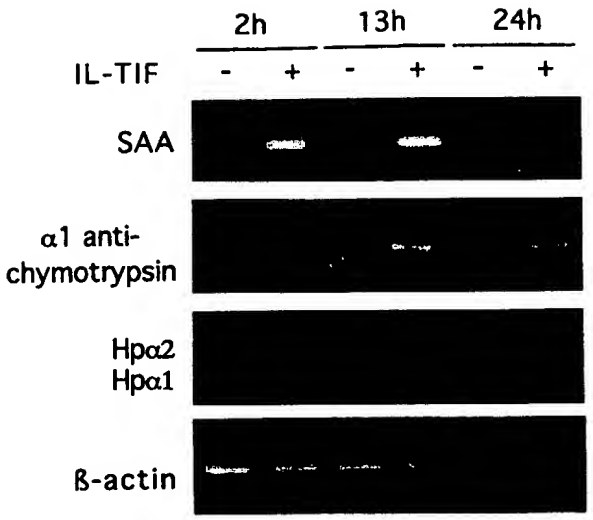


Fig. 3. Induction of acute phase protein by IL-TIF *in vitro*. HepG2 cells were stimulated for the indicated periods of time with hIL-TIF (1% 293-EBNA cell supernatant) or with a supernatant from mock-transfected cells. RT-PCR amplification was performed with oligonucleotides specific for SAA, α1-antichymotrypsin, haptoglobin, and β-actin. PCR products were analyzed by agarose gel electrophoresis. The two bands observed for haptoglobin were sequenced and correspond to previously described allelic variations (23).

block SAA induction, indicating that protein synthesis is not required for hIL-TIF activity. In the presence of cycloheximide, the effect of IL-TIF is even increased. This might result from the inhibition of production of proteins of the SOCS family, which are induced by STAT factors and act as a negative feedback on cytokine activities (9).

Because IL-TIF and IL-6 seemed to have similar activities on acute phase reactants, we addressed the possibility that IL-TIF activity was mediated through gp130. This transmembrane protein is the main signaling component of the IL-6 receptor complex and is shared by other receptors, such as those of leukemia inhibiting factor, IL-11, ciliary neurotrophic factor, and oncostatin M (7). HepG2 cells were stimulated with IL-TIF or IL-6 in the presence of polyclonal anti-gp130 antibodies that block the activity of gp130-interacting cytokines. To quantitatively assess STAT activation, HepG2 cells were transfected with a luciferase reporter construct that included five STAT-binding sites in addition to the TK promoter. As shown in Fig. 5*A*, both IL-TIF and IL-6 stimulated STAT-dependent promoter activity

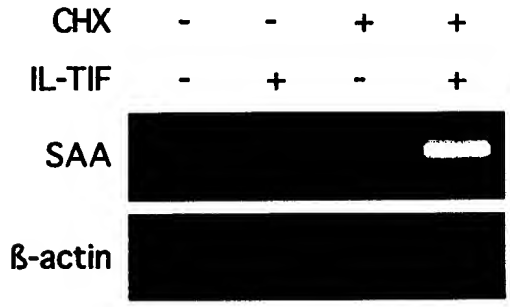


Fig. 4. Protein synthesis is not required for SAA up-regulation by IL-TIF. HepG2 cells were stimulated for 2 h with hIL-TIF (1% supernatant from 293-EBNA cells transfected with the IL-TIF cDNA in pCEP4 expression plasmid) or with a supernatant from mock-transfected cells, and with or without 10 μg/ml cycloheximide. RT-PCR amplification was performed with oligonucleotides specific for SAA and β-actin. PCR products were analyzed by agarose gel electrophoresis.

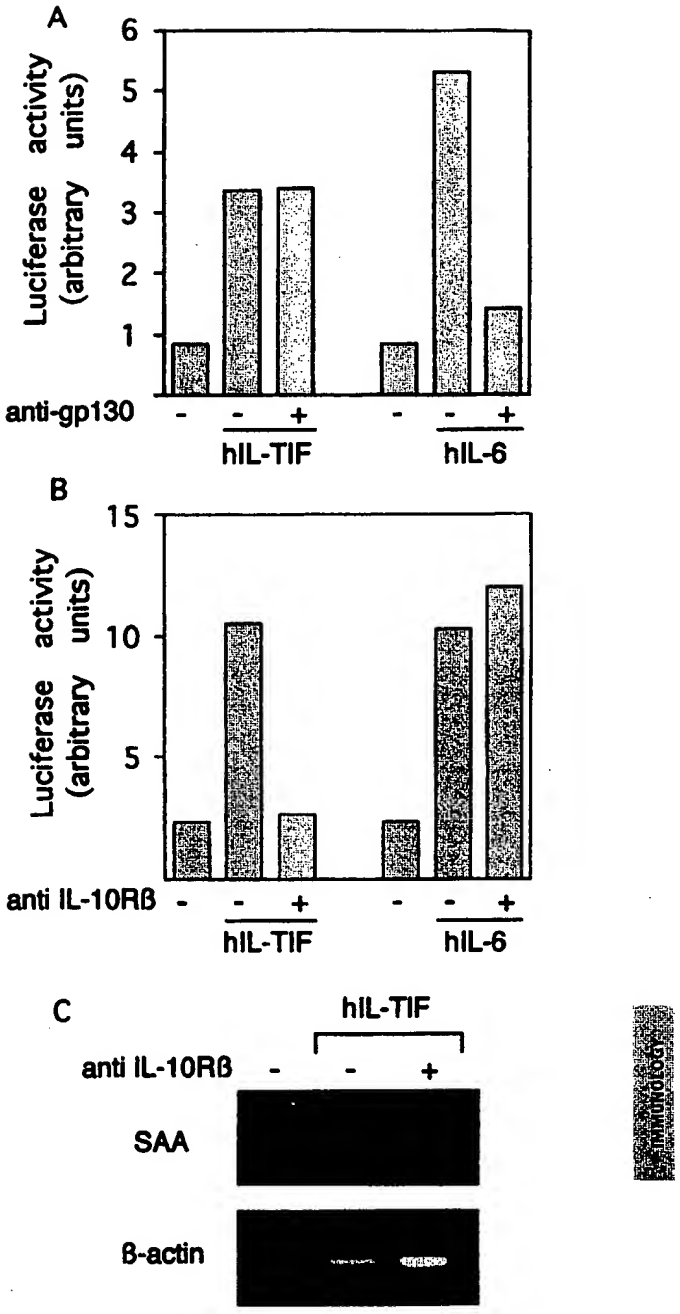


Fig. 5. IL-TIF activity requires IL-10Rβ chain. (*A* and *B*) HepG2 cells, transfected with the pGRR5 luciferase construct, were preincubated with 20 μg/ml anti-gp130 antibodies (*A*) or anti-IL-10Rβ antibodies (*B*) and then stimulated by IL-6 (300 units/ml), IL-TIF (1% supernatants from IL-TIF-transfected HEK293 cells), or left unstimulated. Luciferase activity was monitored 2 h later. The results are expressed in arbitrary units including standardization by using *renilla* luciferase internal control. The data correspond to the mean of duplicate cultures and are representative of three independent experiments. Supernatants from mock-transfected HEK293 cells had no effect in the assay (data not shown). (*C*) HepG2 cells were preincubated with anti-IL-10Rβ antibodies and stimulated with IL-TIF as indicated above. After 2 h, total RNA was extracted and RT-PCR amplification was performed with oligonucleotides specific for SAA and β-actin. PCR products were analyzed by agarose gel electrophoresis.

in HepG2 cells but anti-gp130 antibodies blocked only IL-6 activity. Another candidate as a component of the IL-TIF receptor is the IL-10Rβ chain, which is known to be involved in the IL-10R (10) but whose expression extends beyond the

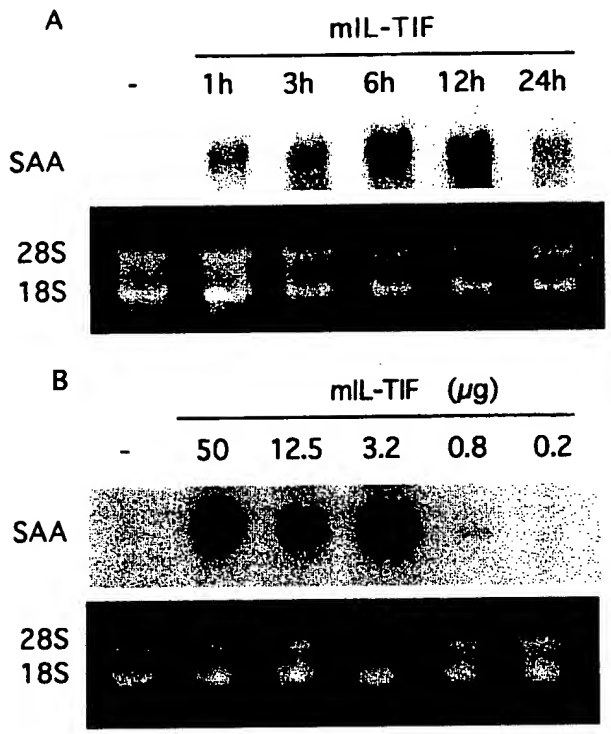


Fig. 6. *In vivo* SAA induction by IL-TIF. (*A*) Kinetics of induction of SAA by IL-TIF *in vivo*. Mice were injected i.p. with 50 μg of recombinant mIL-TIF. Mice were killed after various periods of time, and total RNA was extracted from the liver. Total RNA (10 μg) was fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter before hybridization with the SAA cDNA probe. Ethidium bromide-stained 18S and 28S ribosomal RNA show the equal loading of the gel. (*B*) Dose-dependent induction of SAA by IL-TIF. Mice were injected i.p. with various amounts of recombinant mIL-TIF and were killed 6 h after injection. Total liver RNA (10 μg) was fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter before hybridization with the SAA cDNA probe. Ethidium bromide-stained 18S and 28S ribosomal RNA show the equal loading of the gel.

IL-10-responsive cells. In the presence of anti-IL-10Rβ antibodies, IL-TIF activity was completely blocked whereas that of IL-6 was unaffected, indicating that this receptor chain is indeed involved in IL-TIF signaling (Fig. 5*B*). The same inhibitory effect was observed at the level of SAA expression by HepG2 in response to IL-TIF (Fig. 5*C*), confirming that IL-10Rβ can mediate acute phase reactant induction *in vitro*.

***In Vivo* Activity and Production of IL-TIF.** To assess the ability of IL-TIF to regulate acute phase proteins *in vivo* on normal hepatocytes, recombinant murine IL-TIF was injected i.p. into endotoxin-resistant C3H/HEJ mice. Total RNA was extracted from the liver of these animals, and SAA expression was analyzed by Northern blotting. As shown in Fig. 6*A*, a high dose of mIL-TIF (50 μg) induced SAA expression as soon as 1 h after i.p. injection of the cytokine. The maximal effect was reached after 6 h, and SAA expression decreased at 24 h after injection. Injection of various doses of IL-TIF showed that 3 μg of IL-TIF still resulted in maximal induction, whereas the SAA message was still detectable with 0.8 μg of IL-TIF (Fig. 6*B*). An increase in SAA protein also was detected by ELISA in the serum of mice that received seven daily injections of 30 μg of IL-TIF (data not shown).

IL-TIF was initially identified as a T cell-derived cytokine whereas most cytokines that regulate the liver acute phase response are mainly produced during inflammation. To determine whether IL-TIF also could be produced upon inflammatory stimuli *in vivo*, we injected LPS into BALB/c mice and analyzed IL-TIF mRNA expression after 2 h in various organs. As shown in Fig. 7, LPS injection induced IL-TIF expression in all organs examined, indicating that IL-TIF is actually involved in inflammatory responses.

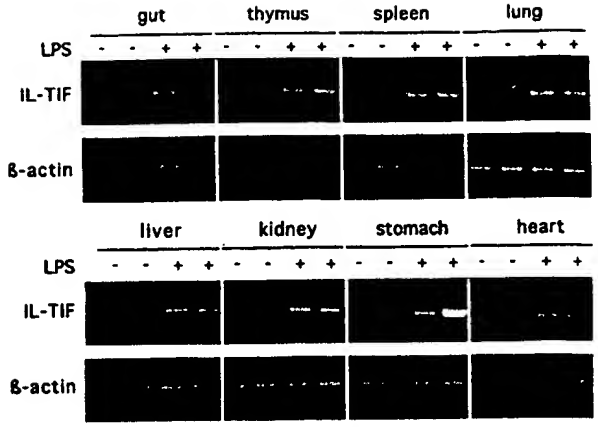


Fig. 7. Inducibility of IL-TIF expression upon LPS injection. BALB/c mice were injected either with 200 μl of 10 μg/ml LPS or the same volume of PBS and were killed 2 h later. Total RNA was prepared from different organs from two mice per condition. RT-PCR amplification was performed with oligonucleotides specific for mIL-TIF and for β-actin. PCR products were analyzed by agarose gel electrophoresis. Similar results were found in three independent experiments.

## Discussion

In the present paper, we report the cloning of the human IL-TIF cDNA and show that this new cytokine is able to up-regulate acute phase reactant production by liver cells. Production of acute phase proteins is considered as a survival mechanism for the short term, but its maintenance for longer periods contributes to chronic inflammation and may have negative clinical consequences (11, 12). This liver response is mainly due to cytokine release by activated macrophages and other cells, particularly IL-1, tumor necrosis factor, and IL-6 (13–16).

The finding that IL-TIF up-regulates acute phase reactant production extends the number of cytokines that might contribute to this process. In addition, we show that the activity of this factor is mediated by IL-10Rβ. This transmembrane protein is required for IL-10 signaling (10), and IL-10Rβ-deficient mice recapitulate the phenotype of IL-10-deficient mice, namely a high susceptibility to inflammatory bowel disease and splenomegaly (17). Our data indicate that this molecule is not only a component of the IL-10 receptor complex but might be shared between a larger family of cytokine receptors for IL-10-related factors. This observation also suggests that further analysis of IL-10Rβ-deficient mice might unmask unique characteristics as compared to IL-10 deficient animals, that could reflect IL-TIF-dependent processes.

In addition, it is likely that other members of the IL-10 family that could also use IL-10Rβ will be described in the near future. Recently, a new cytokine called AK155 was identified because of its up-regulation during viral infection of T lymphocytes (6). This protein shares 27% amino acid identity with IL-10 and shows a similar homology with IL-TIF. Interestingly, the AK155 gene is located on chromosome 12q15, at 40 kb from the IFNγ gene, whereas the IL-TIF gene is located in the very same region, at 90 kb from the IFNγ gene (L.D., unpublished data). This suggests that chromosome 12q15 bears an IFN/IL-10-related cytokine cluster that might be involved in immune and inflammatory responses. In this regard, it must be stressed that chromosome 12q15 has been linked to inflammatory bowel

lisease and to asthma (18–21). Further analysis of polymorphisms in the human IL-TIF gene and the production of IL-TIF-deficient or transgenic animals should allow us to determine precisely the role of this new factor in inflammatory processes.

IL-TIF was originally identified as a gene up-regulated by IL-9 in a murine T cell lymphoma. This suggested that IL-TIF might be responsible for some of the *in vivo* activities of IL-9 such as lymphoma induction, asthma susceptibility, anti-parasite immune response, or B1 lymphocyte expansion (22). Although IL-9 also up-regulated IL-TIF expression in T helper cell clones and mast cell lines *in vitro*, we failed to detect any up-regulation of this gene in IL-9 transgenic mice, suggesting that it does not play a major role in the *in vivo* biological activities of IL-9. However, IL-TIF is produced by normal T cells upon ConA activation in mice, or anti-CD3 stimulation in humans, suggesting a role associated with antigen-specific immune responses. In this report, we show that LPS induces IL-TIF mRNA expression *in vivo* within 2 h in various organs, pointing to other cell types as potential IL-TIF-producers. This finding contrasts with our initial observation that murine spleen cells stimulated for 24 h with LPS *in vitro* failed to express IL-TIF (1). This discrepancy between *in vivo* and *in vitro* IL-TIF induction might reflect an indirect mechanism of gene induction. Further studies will be necessary to elucidate the mechanisms regulating the expression of this new cytokine during inflammatory processes and to determine potential applications of IL-TIF, itself or its antagonists, in modulating inflammation *in vivo*.

**Note Added in Proof.** The human IL-TIF sequence has been recently identified from expressed sequence tag databases by Xie *et al.* (24). It was proposed to rename the protein IL-21.

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REVIEW ARTICLE

# Serum amyloid A, the major vertebrate acute-phase reactant

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The serum amyloid A (SAA) family comprises a number of differentially expressed apolipoproteins, acute-phase SAAs (A-SAAs) and constitutive SAAs (C-SAAs). A-SAAs are major acute-phase reactants, the *in vivo* concentrations of which increase by as much as 1000-fold during inflammation. A-SAA mRNAs or proteins have been identified in all vertebrates investigated to date and are highly conserved. In contrast, C-SAAs are induced minimally, if at all, during the acute-phase response and have only been found in human and mouse. Although the liver is the primary site of synthesis of both A-SAA and C-SAA, extrahepatic production has been reported for most family members in most of the mammalian species studied. *In vitro*, the dramatic induction of A-SAA mRNA in response to pro-inflammatory stimuli is due largely to the synergistic effects of cytokine signaling pathways, principally those of the interleukin-1 and interleukin-6 type cytokines. This induction can be enhanced by glucocorticoids. Studies of the A-SAA promoters in several mammalian species have identified a range of transcription factors that are variously involved in defining both cytokine responsiveness and cell specificity. These include NF-κB, C/EBP, YY1, AP-2, SAF and Sp1. A-SAA is also post-transcriptionally regulated. Although the precise role of A-SAA in host defense during inflammation has not been defined, many potential clinically important functions have been proposed for individual SAA family members. These include involvement in lipid metabolism/transport, induction of extracellular-matrix-degrading enzymes, and chemotactic recruitment of inflammatory cells to sites of inflammation. A-SAA is potentially involved in the pathogenesis of several chronic inflammatory diseases: it is the precursor of the amyloid A protein deposited in amyloid A amyloidosis, and it has also been implicated in the pathogenesis of atheroscelerosis and rheumatoid arthritis.

*Keywords*: acute-phase response; amyloidosis; serum amyloid A; transcriptional regulation.

## THE ACUTE-PHASE RESPONSE

The acute phase response is the immediate set of host inflammatory reactions that counteract challenges such as tissue injury, infection and trauma. Its role is to isolate and neutralize pathogens and prevent further pathogen entry while minimizing tissue damage and promoting repair processes, thereby permitting host homeostatic mechanisms to rapidly restore normal physiological function [1,2]. The acute-phase response involves the induction of an inflammatory mediator cascade which is characterized by both local vascular effects and systemic, multiorgan effects. The latter include biosynthetic changes, particularly pronounced in the liver, which modify the profile of circulating plasma proteins. Although the acute-phase response has evolved as a survival mechanism for the short term, its maintenance over the longer term in cases of chronic inflammation may have negative clinical consequences.

Initiation of the inflammatory cascade occurs primarily through activated blood monocytes and tissue macrophages at the site of the inflammatory stimulus. Upon activation macrophages release a range of primary inflammatory mediators, the most important of which are members of the interleukin-1 (IL-1) and tumor necrosis factor (TNF) cytokine families. These cause the release of a range of secondary cytokines and chemokines (e.g. IL-6, IL-8 and monocyte chemoattractant protein) from local stromal cells. The chemotactic activities of some of these molecules draw leukocytes such as neutrophils to the inflammatory site, where they in turn release further pro-inflammatory cytokines [3]. Consequently, the cytokine cascade and the recruitment of immune effector cells is rapidly augmented locally to deal with both the underlying inflammatory stimulus and the cellular debris generated by any associated tissue damage.

One of the most intensively studied systemic responses to an acute inflammatory stimulus is the alteration in the hepatic biosynthetic profile of acute-phase proteins (APPs) (reviewed in [4,5]). Increased synthesis of the positive APPs which counteract the inflammatory challenge ensures that they attain the plasma concentrations at which they are maximally effective. The positive APPs may be subclassified as either major or minor according to their fold induction during the acute-phase response. Partially to permit synthetic capacity to

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*Abbreviations*: ApoA-I, apolipoprotein A-I; APP, acute-phase protein; A-SAA, acute-phase serum amyloid A; C-SAA, constitutive SAA; ECM, extracellular matrix; HDL, high-density lipoprotein; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IFN-γ, interferon-γ; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; GRU, glucocorticoid-responsive unit; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MM-LDL, minimally modified low-density lipoprotein; rhA-SAA, recombinant human A-SAA; rmIL-1ra, recombinant mouse IL-1ra ; SAF, SAS-binding factor; SAS, SAA-activating sequence; SEF-1, SAA enhancer factor 1; $sPLA_2$, secretory phospholipase $A_2$; TNF, tumor necrosis factor; YY1, yin and yang 1.

*Note*: serum amyloid A nomenclature used in this review follows the new guidelines from the SAA Subcommittee of the Amyloidosis Nomenclature Committee [Sipe, J.D. & Committee (1999) *Amyloid: Int. J. Exp. Clin. Invest.* 6, 67–69] and Table 1.

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be redirected to the increased production of the positive APPs, another distinct class of liver proteins, the negative APPs, are down-regulated during the acute-phase response.

In addition to cytokines and chemokines, other inflammatory mediators include glucocorticoids and growth factors. Glucocorticoids can stimulate the expression of some APPs directly; however, they usually act by enhancing the transcriptional induction that is principally driven by cytokines such as IL-1, TNF and IL-6. Concurrent with their participation in pro-inflammatory processes, IL-1, TNF and IL-6 contribute to the resolution of the acute-phase response by initiating negative feedback on their own production by inducing corticosteroids which, as well as enhancing the cytokine-driven upregulation of APP synthesis, inhibit further cytokine gene expression. This feedback acts via the cytokine-induced production of adrenocorticotrophic hormone which in turn stimulates synthesis of the corticosteroid, cortisol [6–8]. Among the various growth factors that modulate APP regulation, insulin can attenuate the induction of most APP genes by IL-1 and IL-6 type cytokines [9], and is an example of a factor that has the opposite regulatory input to that associated with glucocorticoids.

## THE SERUM AMYLOID A (SAA) FAMILY

The SAA family was originally considered to comprise only a single circulating precursor of the amyloid A protein from which its name is derived. The amyloid A protein is the principal component of the secondary amyloid plaques that may be deposited in major organs as an occasional consequence of chronic inflammatory disease (see below) [10]. The SAA family is now known to contain a number of differentially expressed apolipoproteins which are synthesized primarily by the liver and can be divided into two main classes based on their responsiveness to inflammatory stimuli. 'Acute-phase' serum amyloid A (A-SAA) is the archetypal vertebrate major APP. It is induced from resting levels by more than 1000-fold during inflammation to plasma concentrations that can exceed 1 $mg \cdot mL^{-1}$ [2,11,12], implying an important, beneficial role in host defense. During inflammation, A-SAA associates predominantly with the third fraction of high-density lipoprotein (HDL3) [13] replacing apolipoprotein A-1 (ApoA-1) as the predominant apolipoprotein on this particle [14]. 'Constitutive' SAAs (C-SAAs) have been described in two species, human [15] and mouse [16]. Unlike the A-SAAs, the C-SAAs, which are at most minimally induced during the acute-phase response, are associated with both normal and acute-phase HDL [15–17].

### SAA genes and proteins

Multiple SAA genes and proteins have been described for several mammalian species including human, mouse, hamster, rabbit, dog, mink, cow, sheep and horse. The high degree of conservation of the SAA genes and proteins that has been maintained through the evolution of eutherian mammals [18] extends to other vertebrates including marsupials [19] and fish [20] thereby providing further evidence that they are likely to have important biological functions. All of the SAA genes described to date share a four-exon three-intron organization which is characteristic of many other apolipoproteins [4]. The mature SAA proteins range in size from 104 to 112 amino acids and are derived from primary translation products with 18-amino acid leader peptides (Fig. 1).

The SAA genes of human and mouse, which comprise four and five members, respectively, are those that have been subject to the most comprehensive analyses (see below). In other mammalian species the SAA family members are less well defined. There are at least three transcribed A-SAA genes in dog [21], mink [22,23] and rabbit (for references, see [24,25,26,27]). In the case of horse, three SAA isoforms have been found in acute-phase serum [28,29]. There are also at least

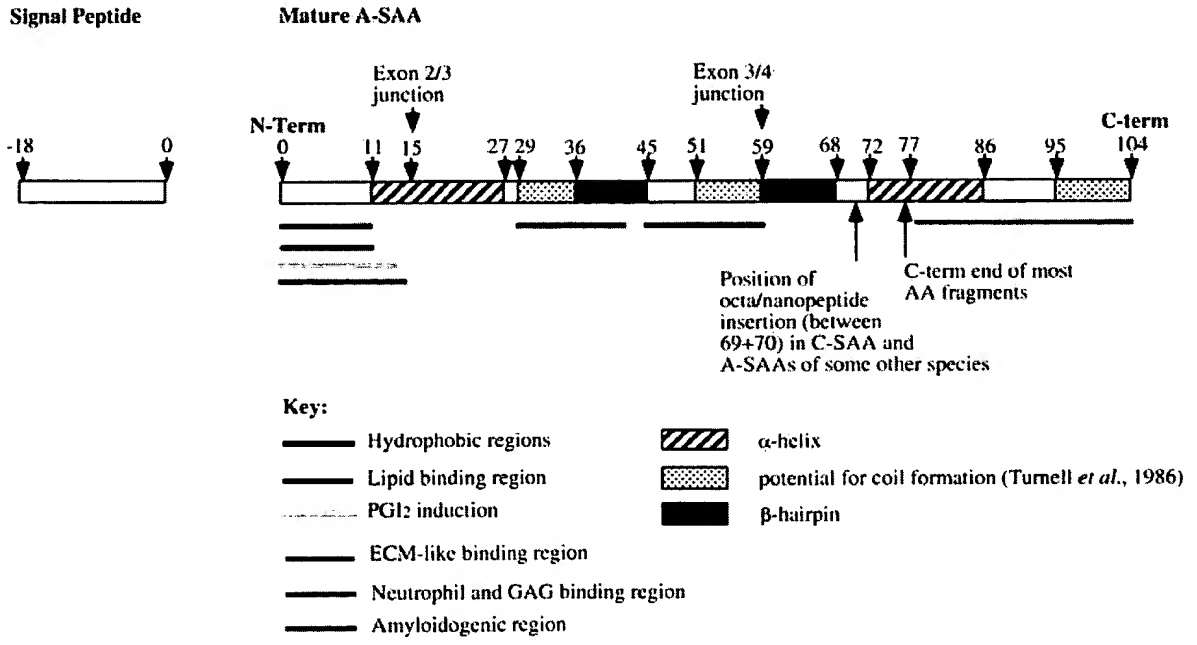


**Fig. 1. The structure of human A-SAA protein.** The 18-amino acid signal peptide is shown (−18 to 0) together with the 104 mature protein (1–104). The most frequently observed C-terminus of the amyloid A protein is indicated at residue 76. Regions of potential functional importance are indicated by underlining in color, while structurally important regions are indicated by shading/hatching; both are discussed in the text. Sites delimiting sequences encoded by exons 2/3 and 3/4 are indicated (exon 1 contains only 5′ untranslated region).

**Table 1. The SAA family.** Nomenclature for the human and mouse SAA families is from the new guidelines of the SAA Subcommittee of the Amyloidosis Nomenclature Committee, 1999 [36]; square brackets represent the old nomenclature for clarity. The SAA family members of other species and their gene/protein numbers are as referred to in original reports.

| Gene | Protein | Alleles (coding) | Alleles (noncoding) | References |
|---|---|---|---|---|
| Human | | | | |
| SAA1 | SAA1 (A-SAA) | SAA1.1 [SAA1α] | SAA1.1.1 [SAA1α1] | [280,281,282] |
| | | | SAA1.1.2 [SAA1α2] | [283] |
| | | | SAA1.1.3 [SAA1α3] | [44] |
| | | SAA1.2 [SAA1β] | | [284] |
| | | SAA1.3 [SAA1γ] | | [276,285] |
| | | SAA1.4 [SAA1δ] | | [286] |
| | | SAA1.5 [SAA1β] | | [287] |
| SAA2 | SAA2 (A-SAA) | SAA2.1 [SAA2α] | SAA2.1.1 [SAA2α1] | [282,287] |
| | | | SAA2.1.2 [SAA2α2] | [44] |
| | | SAA2.2 [SAA2β] | | [288,146] |
| SAA3 | Pseudogene | | | [37,55] |
| SAA4 | SAA4 (C-SAA) | | | [15,288] |
| Mouse | | | | |
| Saa1 [Saa2] | Saa1 [Saa2] (A-SAA) | | | [45] |
| Saa2 [Saa1] | Saa2 [Saa1] (A-SAA) | | | [45] |
| Saa3 | Saa3 | | | [45] |
| Saa4 [Saa5] | Saa4 [Saa5] (C-SAA) | | | [16] |
| Saa-ps1 [Saa4] | Pseudogene | | | [45] |
| Saa2.2 [CE/J Saa] | Saa2.2 | | | [289] |

two A-SAAs in hamster [30,31], and one each in cow [32,33], sheep [34] and wallaby [19]. Rat mRNA has been identified in primary hepatocytes, by representational differential and Northern-blot analyses, in response to aflatoxin B1, a mutagenic and carcinogenic food contaminant (see below) [35]. A-SAA isoforms have also been detected in fox and goat sera [24]. Arctic char (a salmonid closely related to trout), is the most evolutionarily distant vertebrate for which an SAA has been described [20]. Computer-based analysis of their sequences and incorporation into phylogenetic trees indicates that both the wallaby and arctic char proteins are A-SAAs rather than C-SAAs, and *in vivo* studies of the salmonid mRNA have established that its hepatic expression is dramatically induced by inflammation [19,20]. Furthermore, the *in vivo* studies of the hepatic induction of A-SAA mRNA in arctic char challenged with a lethal inoculum of the pathogen *Aeromonas salmonicida* indicate that the level of synthesis is driven by pathogen load. This strongly suggests that the dramatic upregulation of A-SAA in response to inflammatory stimuli is common to all vertebrates and in addition suggests a shared protective biological function.

The four human and five mouse SAA family members were originally named in numerical progression according to the order in which they were identified. Consequently SAA nomenclature in the literature does not accurately define true homologs between the species [18]. In order to address this, a revised nomenclature for the SAA family has recently been adopted [36] and is used in this review (Table 1). The SAA genes of both the human and mouse lineages have remained in close physical linkage (Fig. 2) and it seems likely that the SAA genes will be similarly clustered in the genomes of other species [18]. The human *SAA1*, *2*, *3* and *4* genes are within 150 kb of each other on chromosome 11p15.1 [37,38]. This region of the human genome is syntenic with proximal mouse chromosome 7 [39] to which the mouse SAA genes were originally mapped [40], and within which the position of the cluster, containing *Saa1*, *2*, *3*, *4* and *5* and spanning only 45 kb, has been refined [41,42].

The human *SAA1* and *SAA2* and the mouse *Saa1* and *Saa2* genes all encode A-SAAs. It is impossible to determine the interspecies evolutionary relationships of these individual SAA family members based on nucleotide or protein sequence comparisons alone [18]. However, their relative map positions and transcriptional orientations (Fig. 2) provide strong evidence that human *SAA1* and mouse *Saa1* (formerly *Saa2*) are evolutionary homologs, as are human *SAA2* and mouse *Saa2* [*Saa1*] [38,42]. Within each species these two genes have almost identical sequences and organizations, suggesting that the A-SAA genes have been subjected to recent gene conversion (i.e. homogenizing) events within each evolutionary lineage [18]. They are co-ordinately induced during the acute-phase response and their mature protein sequences share greater than 90% identity. In the case of human, both *SAA1* and *SAA2* are allelic. *SAA1* has at least five alleles [43], of which three (*SAA1.1*, *SAA1.2* and *SAA1.3*) encode distinct proteins, and two are neutral polymorphisms of *SAA1.1* (Table 1). *SAA2* has three alleles, *SAA2.1*, of which there is a *Hin*dIII polymorphism of neutral effect [44], and the distinct *SAA2.2* (Table 1).

Mouse *Saa3* is an expressed *A-SAA* gene although it is more diverged relative to the other *A-SAA* genes *Saa1* and *Saa2* [45–47]. Its protein product shares only 63% and 65% identity with Saa1 and Saa2, respectively. Unlike the other mouse SAAs, expression of Saa3 mRNA is not principally hepatic [48–50] and the Saa3 protein has been identified as a secreted product of macrophages [51]. *SAA3* genes and mRNAs have been reported in other species, e.g. rabbit, rat and hamster, in all of which expression occurs in a range of extrahepatic tissues [30,31,52,53]. In contrast, the human *SAA3* gene, the presumptive evolutionary homolog of mouse *Saa3* [54], is a pseudogene because of a single base insertion in exon 3 which produces a frameshift and, consequently, generates a downstream stop signal at codon 43 [37,55]. No mRNA or protein

product specified by human *SAA3* has been identified. However, it is clear that human SAA3 protein, if it exists, cannot be an 'intact' SAA3 molecule with functions analogous to those of the SAA3 molecules in other species.

The fourth member of the mouse SAA family to be identified was a pseudogene, originally named *Saa4* [45], and now referred to as *Saa-ps1* [36]. It is highly disrupted and contains only sequences related to exons 3 and 4 of the functional SAA genes together with a 25-bp deletion in the exon 3 portion, which introduces an in-frame stop codon downstream. The most recently identified human and mouse SAA genes are those encoding the C-SAAs. The human *SAA4* gene [15], and the mouse gene, originally called *Saa5* [16], are the only constitutively expressed SAA genes described to date. Based on their expression characteristics, sequences and positions within the human and mouse *SAA* gene clusters, they are considered to be evolutionary homologs [16,18,38,42]. Consequently mouse *Saa5* is now referred to as *Saa4* [56]. The human SAA4 protein sequence shares only 53% and 55% identities with human SAA1 and SAA2, respectively [15], and mature mouse Saa4 has 48% amino acid identity with Saa1 and Saa3 [16], establishing that the C-SAAs constitute a distinct branch of the SAA family. Both the human *SAA4* and mouse *Saa4* genes encode precursor molecules of 130 amino acids from which 18-residue signal peptides are cleaved to yield 112-residue mature proteins. The products are therefore eight amino acids longer than the mature A-SAAs in these species because of the apparent 'insertion' of an octapeptide between residues 69 and 70 (Fig. 1). Within the octapeptide encoded by the *SAA4* gene, there is a glycosylation site (NSS) that is differentially used to generate the 14-kDa unglycosylated and 19-kDa glycosylated forms of C-SAA observed in human serum [15]. A similarly positioned octapeptide is also present in the A-SAAs of carnivores (dog and mink) and perissodactyls (horse), and a nonapeptide occurs in artiodactyls (cow and sheep). The distribution of these sequence elements within the SAA family is an evolutionary paradox, the functional significance of which, if any, is unknown [15,18].

## Structure of SAA proteins

Early work based on predictive methods suggested that A-SAA is likely to contain two regions of α-helix in addition to β-sheet regions [57] (Fig. 1), the latter of which are common to all amyloid proteins including amyloid A protein [58]. The likelihood of α-helix being present has recently been augmented by CD studies of a human SAA1-staphylococcal nuclease fusion protein [59]. However, to date, structural studies using advanced methods have not been reported. Nevertheless, several regions of mammalian A-SAA proteins that may be important in facilitating the beneficial role(s) A-SAA may play during inflammation, and/or its pathogenic behavior after over-expression during chronic inflammation, have been identified (Fig. 1).

It has clearly been established that A-SAA is the serum precursor of the amyloid A protein that is found in secondary amyloid deposits by tracking the fate of human A-SAA-HDL introduced into mouse *in vivo* models of amyloidosis [60,61]. Although the precise mechanism is poorly defined, proteolytic cleavage of A-SAA appears to play a central role in

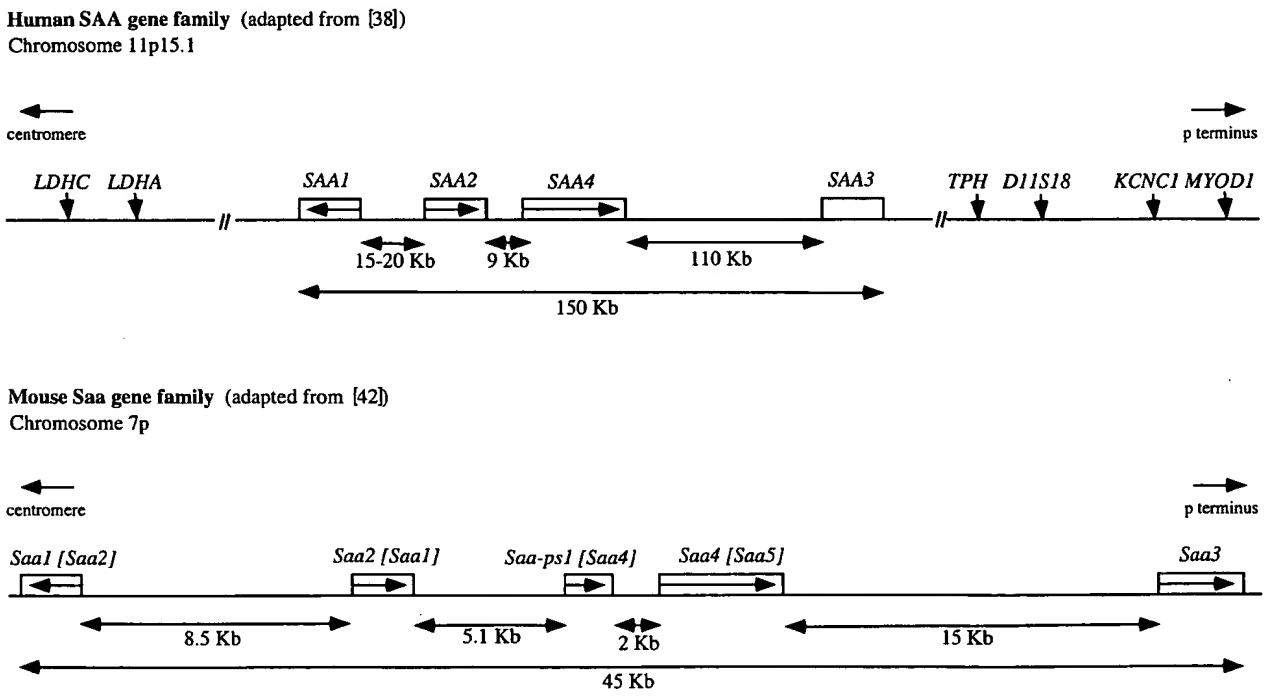


**Fig. 2. A comparative map of the human and mouse SAA gene families.** The human family spans 150 kb on chromosome 11p15.1 [38] while the mouse family spans 45 kb on chromosome 7p [42]; these regions of the human and mouse genome are syntenic. The old SAA nomenclature for the mouse SAA family is in square brackets beside the new (Table 1). The relative positions of the flanking genes lactate dehydrogenase A and C (*LDHA* and *LDHC*), tryptophan hydroxylase (*TPH*), myogenic factor 3 (*MYOD1*) and a member of the potassium channel family (*KCNC1*) are indicated in the case of the human cluster; in mouse, these genes all map to a 500-kb region containing the SAA genes but have not been fine-mapped relative to the SAAs. The position of an anonymous human marker, D11S18, is also shown. Arrows within SAA genes represent 5′ → 3′ orientation of the gene.

amyloid deposition. Such cleavage results in the generation of N-terminal peptides that are deposited as amyloid A protein; these are predominantly 76 residues long, but smaller and larger amyloid A proteins have also been reported [10,62,63]. The capacity of synthetic peptides of human and mouse A-SAA, encompassing amino acids 2–12 and 1–11, respectively, to form fibrils *in vitro* indicates that the amyloidogenic region is within the first 10–15 N-terminal amino acids [64]. Furthermore, site-directed mutagenesis of the eighth amino acid and deletion of the first 11 amino acids of recombinant human SAA1.1 (rhSAA1.1) results in a reduction in amyloid fibril formation [65].

A-SAA and C-SAA both associate with HDL, the former being the major HDL-associated apolipoprotein during the acute-phase response and the latter being the predominant, perhaps only, HDL-associated SAA under normal physiological conditions. The early proposition that the lipid-binding, as well as the amyloidogenic, region of A-SAA resides within the first 11 N-terminal amino acids [57] is consistent with subsequent findings that amyloid A protein which lacks the C-terminal 28 residues, can associate with HDL [66], and that epitopes defined by antibodies raised against N-terminal A-SAA peptides (residues 1–30) are masked when A-SAA is complexed with HDL [67]. Recently, more comprehensive studies have suggested that the epitopes of residues 31–39, 64–78 and 95–104 are exposed whether A-SAA is associated with HDL or not, and epitopes of residues 1–30, 40–63 and 79–94 (the hydrophobic regions of A-SAA) are masked either because of specific self-folding or dimerization; both monomeric and dimeric lipid-free A-SAA coexist in denaturing, non-denaturing, acidic and basic environments [68]. In addition, a reduction in binding to HDL is observed when the N-terminus is mutated by either deletion of the first 11 residues or substitution of the eighth residue (glycine) with aspartate [65].

The region between residues 29 and 42 in human A-SAA contains two elements, YIGSD and RGN, which are very similar to the distinct cell-binding domains of the two extracellular matrix (ECM) cell adhesive glycoproteins, laminin (YIGSR) and fibronectin (RGD), respectively. Synthetic peptides of these elements specifically inhibit the receptor-mediated adhesion of human T lymphocytes and mouse M-4 melanoma cells to laminin and fibronectin, respectively [69]. Amyloid A protein (residues 1–76) and rhA-SAA can also inhibit the adhesion of human T lymphocytes to these two glycoproteins. The primary structure of this rhA-SAA differs from rhSAA1.1, as it has an additional N-terminal methionine and substitutions at positions 60 and 71 which are found in SAA2.2. The above suggests that A-SAA may be able inhibit immune cell migration towards inflammatory sites and, perhaps, metastatic processes *in vivo* [69]. Whereas the YIGSD laminin-like site is found only in human SAA1 and SAA2, the RGN fibronectin-like site is present in all A-SAA and C-SAA proteins described to date (for sequences, see [18,19,20]).

The RGD sequence of the ECM adhesive proteins is also the target that facilitates the binding of some of the mediators released from activated platelets, e.g. fibrinogen, to the platelet integrin glycoprotein IIb–IIIa receptor which is essential in platelet aggregation. It has been suggested that the inhibition of platelet aggregation that can be achieved using A-SAA, residues 25–76 of A-SAA, or amyloid A protein may occur through the conserved RGN sequence [70,71]. However, it should be noted that not all RGD-containing proteins interact with the glycoprotein IIb–IIIa complex and some RGD sequences may not be functionally active. Further evidence of A-SAA involvement in platelet aggregation is provided by its modulation of the induction of prostaglandin $I_2$, a potent antiaggregation agent; this is mediated by the first 14 N-terminal amino acids of A-SAA [72].

A putative calcium-binding sequence, GPGG, between residues 48 and 51 [57], is conserved in all SAA sequences identified to date except for mouse Saa4, in which the corresponding tetrapeptide is GSGG. Early reports that amyloid deposits are rich in calcium [73] and are sites at which $Ca^{2+}$-dependent protein binding interactions occur [74] suggested that this peptide may be important in amyloidogenesis. Furthermore, serum amyloid P, the other major component of secondary amyloid deposits, can bind glycosaminoglycans and amyloid A protein in both $Ca^{2+}$-dependent and $Ca^{2+}$-independent manners [75]. However, a recent report in which the capacity of human SAA1 to enhance the production of thromboxane $A_2$ and prostaglandins can clearly be demonstrated, provided evidence that this property is not $Ca^{2+}$-dependent [76]. In this study, SAA1 was unable to bind $Ca^{2+}$, and, in addition, antibodies specific for residues 40–63 of SAA1, which encompass the GPGG site, had no effect on SAA1-mediated thromboxane $A_2$ and prostaglandin synthesis.

Although relatively few functional studies of the C-terminus of A-SAA have been performed, there is evidence that it may facilitate binding to neutrophils. A peptide corresponding to residues 77–104, but not peptides spanning other regions, competitively inhibits such binding [77]. Interestingly residues 77–104 are usually not present in amyloid A deposits, raising the possibility that they are specifically released after proteolysis of A-SAA and that they have an immune-related biological function. Recently A-SAA has also been shown to have glycosaminoglycan, i.e. heparin sulfate and heparin, binding activity at its C-terminus between residues 77 and 103 [78].

## EXPRESSION AND INDUCTION OF SAA

The major site of A-SAA synthesis, like that of most other acute-phase proteins, is the liver. A-SAA mRNA can be dramatically induced by inflammatory stimuli and may become one of the most abundant hepatic mRNAs [12,46]. In stimulated mice, as much as 2.5% of the synthetic capacity of the liver may be directed to A-SAA protein synthesis [79], while in arctic char infected with a lethal dose of the pathogen *A. salmonicida*, the amount of hepatic A-SAA mRNA present can increase to as much as 10% of the total [20]. A-SAA is also catabolized in the liver [80], has a much shorter half-life of 1 day [81] and is cleared from the plasma more rapidly than other HDL apoproteins such as apolipoprotein A-I (ApoA-I) which has a half-life of 4–6 days [82,83]. During an acute-phase response or chronic inflammation, the capacity of the liver to degrade A-SAA decreases, by 14% and 31%, respectively [84], thereby contributing to the elevated circulating A-SAA levels observed under these conditions.

A-SAA mRNA and protein synthesis are induced *in vivo* during the inflammatory response to challenges such as tissue injury, infection and trauma in all vertebrate species (Fig. 3). These challenges, which can be experimentally produced using agents such as bacterial lipopolysaccharide (LPS), casein, turpentine, $AgNO_3$ and surgery, induce the pro-inflammatory cytokine cascade. The principal cytokines involved in the induction of A-SAA are IL-1, TNF-α and IL-6 (see below). Other cytokines that may be involved either directly or indirectly in A-SAA induction include IL-2, interferon-γ (IFN-γ) and ciliary neutrophic factor [85–87]. In addition to

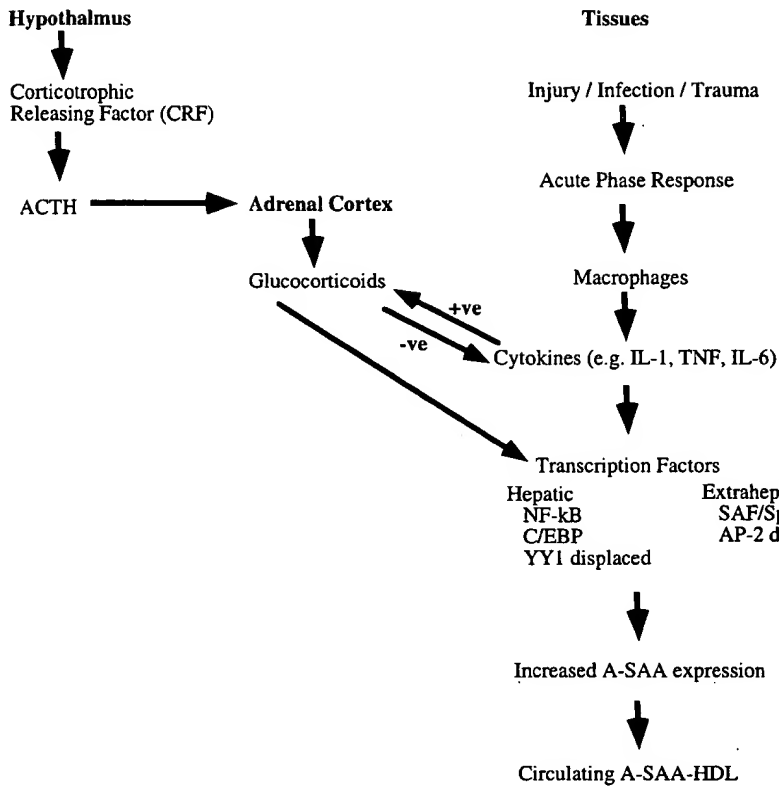


**Fig. 3. Induction of A-SAA during the acute-phase response.** The flow diagram represents the induction of the acute-phase response in tissues by inflammatory stimuli leading to recruitment of macrophages and subsequent cytokine production. These cause changes in transcription factor availability in different tissues which result in increased A-SAA expression and consequently increased protein concentrations. The production of glucocorticoids by the adrenal cortex is also shown; these are upregulated by inflammatory cytokines and enhance A-SAA synthesis, but themselves downregulate the systemic acute-phase response. ACTH, corticotropin.

IL-6 and ciliary neutrophic factor, four other IL-6 type cytokines, i.e. IL-11, leukemia inhibitory factor, oncostatin M and cardiotrophin-1, induce A-SAA when administered to mice [88]. Glucocorticoids, which are also released during inflammation, have been shown to enhance cytokine-induced A-SAA expression in several of the above studies (see below).

Although the liver is the major site of APP synthesis, the extrahepatic tissue/cellular expression of a wide range of APPs has been documented [89]. In most mammalian species, SAA1 and SAA2 are predominantly synthesized in the liver while SAA3 is the main isoform expressed at extrahepatic sites [10]. In mouse, for example, the extrahepatic synthesis of Saa2 has only been definitively documented in the kidney and intestine, while that of Saa1 appears to be restricted to the kidney [49]. Saa3, however, is produced in a wide range of tissues and cell types including macrophages [48,50,51,90]. Mouse A-SAA mRNA has also been detected by *in situ* hybridization in unstimulated and LPS-stimulated intestinal and liver epithelial cells and in stimulated convoluted tubules of the kidney [91], and by Northern-blot analyses in both mouse bone marrow stromal cells following induction by either TNF or IL-1 [92] and the jejunal mucosa in response to LPS-induced endotoxemia [93]. This last study also showed, by immunohistochemistry, that A-SAA protein expression is mainly in the lamina propria cells of the mucosa and the intestinal lumen. In all of the last three studies, the detection methods used could not identify which specific A-SAA isoform or combination of isoforms was expressed. In contrast with constitutive human SAA4 (below), mouse Saa4 mRNA has not been found extrahepatically [16]. In rats, SAA3 mRNA, but not the SAA1/2 mRNA, has been found extrahepatically following LPS, but not turpentine, treatment in the lung, ileum and large intestine [53]. However, in a third rodent species, i.e. hamster, SAA1, SAA2 and SAA3 mRNAs have been found in a wide range of tissues including kidney, stomach, muscle, spleen, brain, heart, lung, intestine, ovary, testis and uterus [30,94].

In mink, extrahepatic SAA gene expression has also been reported in both unstimulated ovary, testis, brain and intestine and in LPS-induced lung, heart, kidney convoluted tubules and uterine endometrium epithelial cells. SAA expression was also observed in amyloidotic adrenal glands. Again the transcripts do not appear to be derived from either SAA1 or SAA2 but from a third, distinct, mRNA species [23,91]. Of the rabbit SAA members, only SAA3 mRNA has been found in a wide range of extrahepatic tissue and cell types [91,95], including, significantly, synovial fibroblasts (see below) [52]. The induction profiles of the three rabbit SAA mRNAs in the liver, and of rabbit SAA3 mRNA in other tissues, vary considerably with different inflammatory agents [95].

Although initial reports of extrahepatic SAA expression were mainly for species other than humans, human extrahepatic SAA expression has recently been documented. In humans, both A-SAA (SAA1 and SAA2) and C-SAA (SAA4) mRNAs are expressed in monocytic/macrophage cell lines, including THP-1 cells [96,97]. SAA mRNA has also been detected in various cell types in human atherosclerotic lesions (i.e. macrophages, endothelial and smooth muscle cells) and in adipocytes using an RNA probe with 81% and 71% homology to human SAA1/2 and SAA4, respectively [98]. Furthermore, cultured smooth muscle cells stimulated with IL-1 or IL-6 plus dexamethasone express SAA1, SAA2 and SAA4 mRNA [98], and both SAA1/2 and SAA4 proteins have been found in atherosclerotic lesions, particularly in foam cells [99]. SAA1/2 and SAA4 mRNAs have also been found in a wide range of non-hepatic cell lines using Northern-blot analysis [100]. More recently, a study employing non-radioactive *in situ* hybridization and immunohistochemical staining has confirmed the colocalization of extrahepatic SAA mRNA expression and

protein production, in a wide range of histologically normal human tissues [101]. This was especially evident in the epithelium of the tissues but was also observed, to a lesser extent, in scattered lymphocytes and plasma cells in the stroma, and expression was also found in the endothelial cells lining blood vessels. More specifically, reverse transcription–PCR of breast (lobule and duct epithelium), large intestine, esophagus, kidney and spleen demonstrated expression of *SAA1*, *SAA2* and *SAA4* genes but not *SAA3*. These findings suggest a possible role for constitutively expressed A-SAA as an immunological defense molecule at local sites, thereby providing an immediate localized defense against inflammatory challenges during the time taken to mount a systemic response by increased hepatic synthesis.

APPs may be designated as either 'type-1' or 'type-2' according to their responsiveness to different classes of cytokines [1]. Type-1 APPs are induced by the IL-1-type cytokines which, in addition to IL-1α and IL-1β, include TNF-α and TNF-β. Type-1 APPs are generally synergistically induced when an IL-1-type cytokine is combined with an IL-6-type cytokine. Type-2 APPs are primarily induced by the IL-6-type cytokines which include IL-6, IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor and cardiotrophin-1. These cytokines all elicit similar responses because of the association of their respective individual receptors with a common membrane-spanning signal-transduction molecule, gp130 (reviewed in [102]). Although IL-6-type cytokines are the main inducers of type-2 APPs, they can also stimulate most other APPs [1]. However, the induction of type-2 APPs by IL-6-type cytokines is not synergistically increased by IL-1-type cytokines; indeed, the latter generally have no effect on type-2 APP biosynthesis, and may even inhibit it.

A-SAA is classified as a type-1 APP as it can readily be induced by either IL-1β or TNF-α. Furthermore, each of these cytokines can synergize with IL-6 to dramatically increase A-SAA mRNA and protein synthesis in several human hepatoma cell lines including PLC/PRF/5, Hep3B and HepG2 [103–114]. In PLC/PRF/5 hepatoma cells, however, IL-6 alone only induces A-SAA mRNA and protein to levels that are barely detectable [108,115,116]. TNF-α induces A-SAA mRNA synthesis in mouse liver *in vivo* [103] and stimulates A-SAA protein synthesis in primary human hepatocytes [117]. Furthermore, there appears to be a significant correlation between TNF-α levels and A-SAA concentrations in patients with sepsis [118]. In PLC/PRF/5 cells the combination of IL-1β and IL-6 is as effective at inducing A-SAA mRNA and protein synthesis as a more complex physiological stimulus, monocyte-conditioned medium [115].

Some caution in assessing the physiological relevance of tissue culture studies should, however, be used as there is considerable heterogeneity between cell lines with respect to the induction of A-SAA by various cytokines. For example, in one study, IL-1β and IL-6, but not TNF-α, could each induce A-SAA protein synthesis in human primary hepatocytes; IL-6 was the most effective inducer but synergy with either IL-1β or TNF-α was not seen [116]. In contrast, others have reported considerable A-SAA protein induction in primary hepatocytes in response to IL-6 but not to IL-1β or TNF-α [119].

In addition to the heterogeneity that exists between the hepatic primary and established cell lines, all of the above investigations used simultaneous cytokine additions when combination treatments were analyzed. Such regimens do not mimic *in vivo* inflammatory situations where the sequential appearance of IL-1 followed by IL-6 [120,121] and TNF-α followed by IL-6 [118] have been demonstrated. Moreover, IL-6, which is induced by both IL-1 and TNF, suppresses both *in vitro* and *in vivo* production of these two cytokines [122]. Furthermore, stimulation with IL-1β before IL-6 is essential for maximal synergistic transcriptional induction of the human *SAA2* promoter *in vitro* [123]. Although IL-6 is required to achieve maximal synergistic induction of *SAA2*, the reciprocal treatment, i.e. stimulation with IL-6 before IL-1β, results in significantly less synergistic activation of the *SAA2* promoter. As well as temporal differences, variable cytokine levels have also been reported in human disease. For example, although A-SAA concentrations correlate with elevated TNF-α levels, IL-6 levels vary, in sepsis patients [118]. In contrast, in Castleman's disease, which is characterized by abnormally high levels of IL-6 production and may lead to systemic reactive amyloidosis, both IL-6 and A-SAA serum levels are high, although both IL-1 and TNF are undetectable [124]. The above suggest that *in vitro* studies of A-SAA expression (see below), and other APP induction, using combinations of cytokines should be designed to accurately reflect the sequential mobilization of individual cytokines at different stages of the *in vivo* inflammatory cascade.

As mentioned above, A-SAA mRNA and protein synthesis can by induced by a variety of pro-inflammatory stimuli. Two of the principal inflammatory agents used in many of the studies of A-SAA expression engage different cytokines, i.e. LPS, which has systemic inflammatory effects, and turpentine, subcutaneous injection of which induces localized tissue damage. The major receptor for LPS on monocytes and neutrophils is CD14. Although interaction of LPS with CD14 leads to IL-1, TNF and IL-6 induction, this ligand receptor interaction does not appear to be necessary for the induction of APPs, including A-SAA, as they can be induced with LPS in CD14-deficient mice to levels comparable with those observed in wild-type mice [125]. Studies of cytokine knockout mice have been used to investigate the complexity of APP induction during the acute-phase response to LPS and turpentine [126–130].

Several studies of transgenic and knockout mice have underscored the complexity of the inflammatory processes involved in A-SAA induction. In response to LPS, IL-1β-deficient mice are able to mount an acute-phase response, including the induction of A-SAA, presumably as they retain a spectrum of cytokines, such as IL-1α, TNF-α and IL-6, that are quantitatively and qualitatively sufficient to produce a systemic signaling cascade as potent as that of wild-type mice [128]. In contrast, these mice do not produce A-SAA protein in response to subcutaneous turpentine injection, indicating that cytokines capable of substituting for IL-1β are not generated by this stimulus [129]. Similarly, IL-6-deficient mice are unable to mount a response to turpentine that is comparable with that of wild-type mice; in particular, these mice did not have elevated liver A-SAA mRNA or serum A-SAA protein levels. However, IL-6-deficient mice are not greatly compromised with respect to their capacity to mount an acute-phase response to LPS [126,127]. TNFα-deficient mice have no difference in serum A-SAA protein induction levels following turpentine treatment when compared with wild-type mice [130]. However, in either TNFα deficient or IL-6 deficient mice, A-SAA serum protein levels could not be induced by LPS to the levels seen in the wild-type, and in mice deficient in both TNFα and IL-6, A-SAA induction was almost completely absent. In contrast, A-SAA mRNA was enhanced after 24 h treatment with LPS in wild-type mice and those deficient in TNFα, IL-6 and TNFα plus IL-6, suggesting post-transcriptional regulation of A-SAA

expression (see below). These results reinforce the likely mechanistic differences between an acute-phase reaction driven by infection as opposed to trauma.

IL-1α and IL-1β can each bind to two distinct receptors, IL-1RI and IL-1RII, both members of the Ig superfamily (reviewed in [131]), of which only the former is able to transduce a signal. A naturally occurring antagonist of IL-1, the IL-1 receptor antagonist (IL-1ra) (reviewed in [132]), inhibits IL-1 by direct competition for IL-1RI, which it binds more effectively than it does IL-1RII, resulting in no signal transduction [133–135]. Two forms of IL-1ra are produced by the same gene and differ only in their N-terminal sequences because of the use of alternative first exons [136]. The secreted form [137,138] is produced by monocytes, macrophages, neutrophils and fibroblasts, but apparently not by hepatocytes and endothelial cells [132]. The alternatively spliced intracellular form is a fully active protein [139,140], the precise function of which has yet to be elucidated. In mice undergoing experimentally induced inflammation the peak of secreted IL-1ra induction coincides with the highest APP concentrations achieved [141]. Consequently there is an integrated biological response during inflammation whereby downregulators of the acute-phase response are induced at the same time as the proinflammatory APPs. In addition to IL-1ra there is a second means of controlling IL-1 agonist activity, i.e. the IL-1RII which may serve as a membrane bound-trap or receptor decoy for IL-1 [142].

IL-1ra has been used to demonstrate the central role that IL-1 plays in A-SAA induction in several studies. It can effect a 75% reduction in the A-SAA protein synthesis induced by monocyte-conditioned medium in human hepatoma cells [109], and can specifically block the IL-1β-driven component of the synergistic transcriptional activation of the human *SAA2* promoter by a combination of IL-1β and IL-6 (see below) [114]. In a BALB/c mouse model of casein-induced acute inflammation, hepatic A-SAA mRNA induction, at least initially, is entirely IL-1-dependent as recombinant mouse IL-1ra (rmIL-1ra), coadministered with the stimulus, completely abolishes the induction of Saa1 and Saa2 mRNA for up to 12 h [143]. In this model, very modest levels of circulating A-SAA protein are observed in the rmIL-1ra-treated mice, indicating that there is extrahepatic synthesis of A-SAA protein that is not exclusively driven by IL-1. Similarly in C57BL/6 mice in which silver nitrate has been used to induce an acute-phase response, coadministration of rmIL-1ra significantly reduces the magnitude of hepatic induction of Saa1 and Saa2 mRNA for up to 24 h [144]. In the latter case, however, rmIL-1ra intervention is qualitatively less effective at reducing serum Saa1 and Saa2 protein, most likely because the more extreme necrotizing nature of the stimulus probably mobilizes a more aggressive cytokine response that permits a significant level of A-SAA hepatic synthesis to occur.

## TRANSCRIPTIONAL REGULATION OF SAA

A number of studies of the human *SAA2* [112,145–149], mouse *Saa3* [150–153], rat *SAA1* [154–157] and rabbit *SAA2* [97,113,158–164] acute-phase promoters have been carried out to identify the *cis*-acting sequences and *trans*-activating transcription factors involved in the massive transcriptional upregulation of the A-SAAs following an acute-phase stimulus. In the A-SAA promoters of all four species, NF-κB and C/EBP transcription factor recognition sequences (reviewed in [165,166], respectively), which are involved in conferring cytokine responsiveness and/or cell specificity of expression on genes, have been located and characterized. Co-operation between members of these two transcription factor families, which associate with each other via their bZIP and Rel domains, respectively [167,168], appears to be important for the induction of A-SAA gene transcription. In addition, several other transcription factors have been implicated in the regulation of A-SAA gene expression (see below).

### Human *SAA2* promoter

Early experiments in which a human *SAA2* genomic clone was transiently transfected into mouse L cells established that A-SAA expression is enhanced by IL-1β, TNF-α, IL-6 and IFN-γ [146,147]. Subsequent analysis of the activity retained by *SAA2* promoter deletion constructs fused to a CAT reporter gene, together with electrophoretic mobility shift and footprinting assays in the human hepatic HepG2 cell line, identified several transcription-factor-binding sites (Fig. 4) [112,145,148]. A proximal NF-κB element, located between residues −91 and −82, is necessary for constitutive expression, and also for full responsiveness of the promoter to IL-1β and IL-6 when either is used alone. In addition, it is needed for the synergistic activation that is apparent when these two cytokines are used in combination. IL-1β can induce binding, maximal at 5–30 min after induction, of a NF-κB-like protein [145] that is similar to the constitutive H2TF1 transcription factor [KBF1 or p(50)2] [169]. Although IL-6 does not directly induce transcription factor binding to this site, it has a reduced capacity to activate the *SAA2* promoter when the site is mutated [112]. Thus it is likely that there is some co-operativity between transcription factors and/or target sites such that the IL-6-mediated effect interacts with the proximal NF-κB site.

A C/EBP site (−184 to −171), identified by methylation-interference footprinting, is also required for the full response to either IL-1β or IL-6 administered alone, and to generate the maximum synergistic activation that can be achieved by combining these two cytokines [112]. IL-6 alone and the IL-1β plus IL-6 combination both induce similar changes in the pattern of factor binding to this site from 15 min through to 16 h after treatment. Binding can be prevented by preincubation of IL-6-treated nuclear extracts with antiserum against C/EBPβ (NF-IL6), indicating that the transcription factors are C/EBPβ or members of the C/EBP family.

Nuclear extracts from cells treated with either IL-1β alone or IL-1β plus IL-6 can interact with synthetic NF-κB targets to form NF-κB bandshift complexes that can be detected within 15 min of treatment and that subsequently decrease quantitatively through to 16 h [112]. Antibody against the p50 subunit of NF-κB can supershift these complexes, establishing that they are at least partly composed of NF-κB p50. A constitutive factor that does not react with anti-p50 can also be detected; this is not affected by cytokine treatment but may compete with NF-κB for the site, as its binding is enhanced by mutating the site to preclude NF-κB binding. Co-transfection experiments with NF-κB subunit (p50, p52 and p65) expression vectors indicate that co-operativity between the p65 subunit and C/EBPβ are largely responsible for the synergistic induction of the *SAA2* promoter. This may be due to a direct association between p65 and C/EBPβ, as has been demonstrated for p50 [167], or p65 may interact via endogenous p50 bound to the proximal NF-κB site.

More recently, electrophoretic mobility-shift assays with HepG2 nuclear extracts have shown that NF-κB p65 and p50, but not p52 or C-Rel, can bind the proximal NF-κB site in response to IL-1 and that C/EBPβ and C/EBPδ, but not CEBPα,

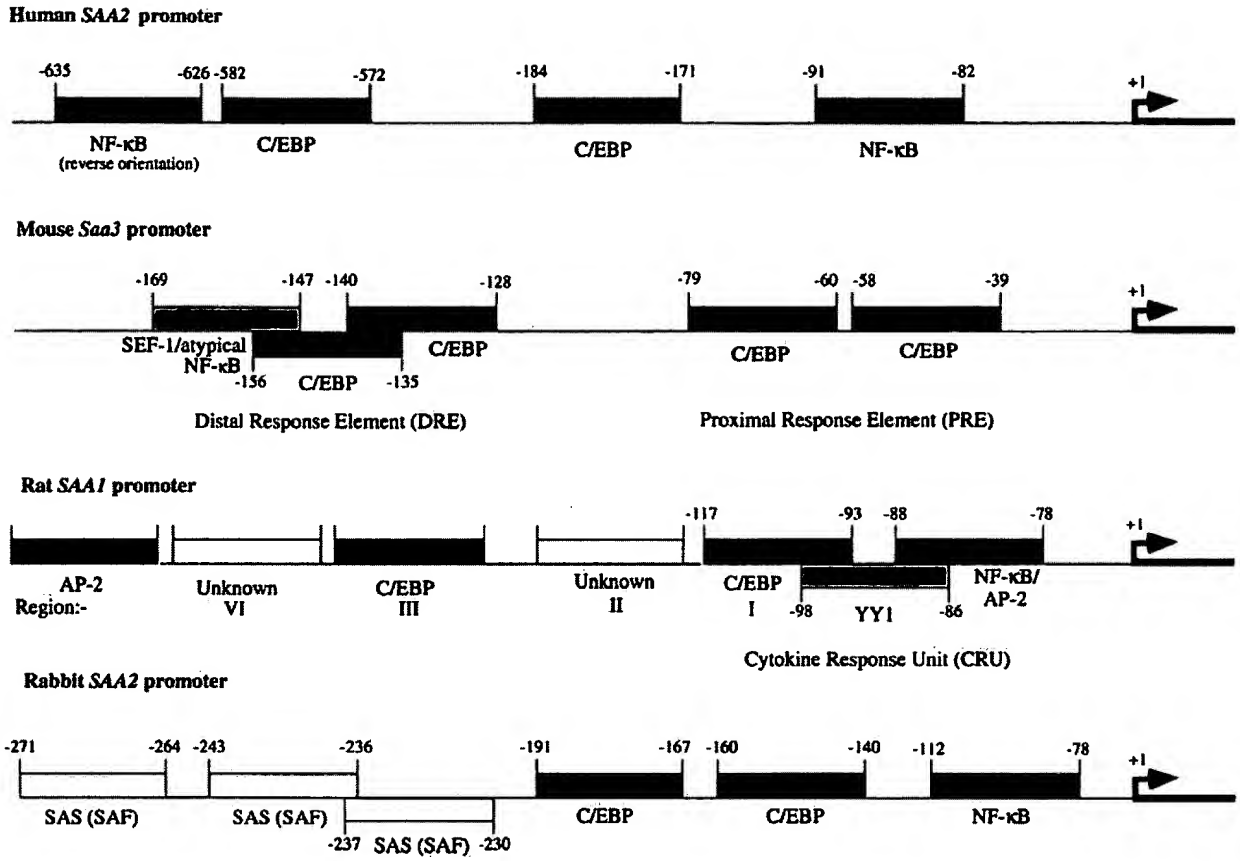


**Fig. 4. Regulatory elements of mammalian A-SAA promoters.** The relative positions of transcription-factor-binding regions of the human *SAA2*, mouse *Saa3*, rat *SAA1* and rabbit *SAA2* promoters are shown and are discussed in the text. The transcription start sites of the genes are indicated by +1 and an arrow. Transcription-factor-recognition sites are NF-kB, C/EBP, AP-2, SAS, which binds SAF, SEF-1 and YY1. In the case of the rabbit, Sp1 has been shown to affect the promoter through its association with SAF.

bind the C/EBP site in response to IL-6 [149]. Co-expression of NF-κB p65 with C/EBPβ and C/EBPδ showed co-operative association of the former with both C/EBP proteins; this association appears to be through direct protein to protein interaction. Mutation analysis of NF-κB p65 suggests that the C-terminal regions of both the Rel homology and the activation domains are important for the interaction with C/EBPβ [149].

In addition to the NF-κB and C/EBP sites described above, the human *SAA2* promoter has a distal NF-κB site between residues −635 and −626, which is in reverse orientation to the proximal NF-κB site, and a 3′ adjacent C/EBP site between residues −582 and −572 (145), both of which appear to lie in an upstream repressor element. Mutation of the distal NF-κB site permits a doubling of both constitutive and IL-1β-stimulated expression. A constitutive nuclear factor with presumptive repressor activity, which binds the region spanning the NF-κB and adjacent C/EBP sites, is displaced when an IL-1β-inducible NF-κB-like factor binds to the NF-κB site. The constitutive factor may be a member of a family of constitutive C/EBP-like negative factors that interfere with IL-1-inducible binding of positive NF-κB-like factors [170].

The kinetics of human A-SAA promoter engagement are being investigated by us using a construct containing 1.2 kb of the human *SAA2* upstream region fused to a luciferase reporter gene [114,123]. When transiently transfected into HepG2 cells, the construct can be effectively stimulated by inflammatory mediators [114], in a manner that closely mimics that of the endogenous *SAA2* gene in established hepatoma culture systems [112,115]. The level of induction of *SAA2*-promoter-driven transcription by the IL-1-type cytokines, IL-1β and TNF-α, increases with time. In contrast, IL-6 has its greatest effect at the early time points and induces progressively less transcription at later time points, thus establishing that the kinetics of promoter engagement by the IL-1-type and IL-6-type cytokines are quite distinct. Combined agonist treatments, i.e. IL-1β plus IL-6 and TNF-α plus IL-6, attain levels of transcriptional induction at least 10-fold higher than single cytokine treatments; however, the kinetics of transcriptional induction achieved are qualitatively similar to IL-6 alone (i.e. both are greater at early time points). This indicates that IL-6 is integral to the early massive synergy observed when present with the IL-1-type cytokines, and that it may be the dominant partner in driving changes in promoter activity [114]. To achieve maximal synergistic induction of the *SAA2* promoter, the order of cytokine addition is important; IL-1β followed by IL-6 is much more effective than the reciprocal order [123]. Consequently, the relative timing of exposure of the *SAA2* promoter to specific signals generated by individual cytokines constitutes an additional important level of control. Furthermore, on the evidence available to date, maximum *in vitro*

effects are produced by addition of cytokines in the order that reflects their appearance in the *in vivo* inflammatory cascade (see above).

### Mouse *Saa3* promoter and the SAA enhancer factor (SEF-1)

The 350-bp region immediately upstream from the transcription-start site of the mouse *Saa3* gene is sufficient for both cytokine-induced and cell-specific expression of the gene [150]. Within this section there is a proximal response element between residues −39 and −79 which is necessary for hepatic expression of the gene and which contains two C/EBP-binding sites (Fig. 4) [151]. There is also a distal response element between −128 and −169 that is required for responses to conditioned medium and IL-1α [150]. The distal response element consists of three binding regions, all of which are required for full cytokine induction. Two of these are C/EBP sites with different binding affinities and the third site binds a novel constitutive transcription factor, the SAA enhancer factor 1 (SEF-1) [152]. As in the case of the rabbit *SAA2* promoter (see below), C/EBP-β and C/EBP-δ bind the mouse *Saa3* C/EBP sites with highest affinity in the induced state. NF-κB also interacts with an atypical motif, between residues −155 and −164, within the region encompassing the distal response element [153]. NF-κB p65, but not p50, can transactivate the *Saa3* promoter while coexpression of either with C/EBP family members results in efficient induction of the promoter, indicating that synergy between these two transcription factor families occurs in mouse *Saa3* gene transcription [153].

### Rat *SAA1* promoter and the yin and yang (YY1) transcription factor

The rat *SAA1* gene has five known *cis*-regulatory elements within the 304 bp immediately upstream of its transcription start site (Fig. 4) [154,155]. This region responds to conditioned medium and IL-1 in a cell-type-specific manner, i.e. transcription driven by the rat *SAA1* promoter appears to be restricted to liver cells. The most distal three sites, which include a C/EBP site, are required to mediate a higher basal activity of the promoter but are not essential for cytokine responsiveness. A more proximal 66-bp section which contains the remaining two sites is sufficient to confer cytokine responsiveness in a no-cell-specific manner. This cytokine response unit contains a second C/EBP site, with a lower binding affinity than the upstream site, and a NF-κB recognition site; both of these are required for cytokine responsiveness and they interact co-operatively to induce gene expression [154,155].

A third transcription factor, YY1, binds the rat *SAA1* cytokine response unit in hepatic cells [156]. YY1 is a zinc finger protein that is expressed ubiquitously in mammalian cells and either represses or activates gene transcription depending on the particular gene promoter and its cellular environment [171]. Both basal and cytokine-mediated rat *SAA1* expression are repressed by YY1, which competes with NF-κB for overlapping binding sites in the cytokine response unit [156]. Rat *SAA1* gene transcription appears to be determined by the ratio of YY1 to NF-κB rather than changes in the relative binding activity of YY1, as this remains constant after induction by conditioned medium. YY1 is always present in the nucleus and is therefore able to bind and repress the rat *SAA1* promoter constitutively. In contrast, most of the cellular pool of NF-κB is held inactive in the cytoplasm in a complex with its inhibitor, IκB. After an inflammatory stimulus, IκB is phosphorylated and subsequently degraded to release NF-κB which can then translocate to the nucleus (reviewed in [165]). Consequently, the concentration of active NF-κB in the nucleus rises dramatically while YY1 levels remain constant, YY1 is displaced from the *SAA1* promoter and the gene is transcriptionally induced. When signals originating with the underlying pro-inflammatory stimulus cease, active nuclear NF-κB levels drop to permit YY1 to re-establish its interaction with the promoter to repress transcription. In addition to direct DNA binding, YY1 may also function through interactions with other proteins; therefore, C/EBP, in addition to NF-κB, may be required to displace YY1 from the rat promoter *in vivo*.

Recently the tissue-specific transcription factor AP-2 has been shown to interact with two sites in the rat *SAA1* promoter, one of which, like the YY1 site, overlaps the NF-κB site [157]. AP-2 acts as a dominant inhibitor of *SAA1* gene transcription, as it can displace NF-κB from the promoter but not vice versa. As its expression is absent in liver cells and it appears to act as an inhibitor of SAA gene expression in non-hepatic cells, AP-2 may be a key component in defining tissue-specific SAA expression in response to various cytokine combinations under different pro-inflammatory conditions.

### Rabbit *SAA2* promoter and the SAA-activating sequence (SAS)-binding factor (SAF)

The rabbit *SAA2* promoter has two adjacent functional C/EBP-binding elements in addition to an NF-κB element in its promoter region (Fig. 4). Initial studies showed C/EBP-β, C/EBPδ and NF-κB p65 to be involved in the cytokine induction of this gene in the liver [158,161,172]. Activated p65 and C/EBP form heterodimers that associate with the three binding elements to varying degrees; these heterodimers are more potent inducers of transcription than either factor alone and promote transcription via both NF-κB and C/EBP sites [161]. More recently it has been reported that the two pro-inflammatory agents, LPS and turpentine, differentially activate, both quantitatively and qualitatively, the transcription factors that interact with the rabbit *SAA2* promoter [159]. Turpentine, the more potent inducer of A-SAA, activates higher levels of C/EBP for longer periods of time than LPS. However, it does not activate NF-κB, unlike LPS which activates p65. Increased levels of C/EBPβ and C/EBPδ and decreased levels of C/EBPα relative to those present in the uninduced state appear to be important in the induction of rabbit A-SAA [158,159]. This is consistent with the finding that, during an LPS-induced acute-phase response in 2-month-old mice, rapid dramatic increases in C/EBPβ and C/EBPδ mRNA expression are observed while C/EBPα is downregulated [173]. However, C/EBPα is known to play an important role in the acute-phase response of neonatal mice (0–3-h-old); wild-type mice injected with LPS exhibit a strong acute-phase response which includes increased A-SAA gene expression, which is not evident in LPS-injected C/EBPα knockout mice despite the latter having similar high levels of C/EBPβ and C/EBPδ mRNA and DNA-binding activities [174]. Consequently, age-dependent factors may determine the capacity of an individual to respond to any given stimulus and may turn out to be important in the pathologies associated with chronic inflammation.

Treatment with IL-6 causes different transcription factors to be induced in hepatic and non-hepatic cell lines [113]. In the human hepatic cell line HepG2, IL-6 only induces C/EBP-binding activity, particularly that of heterodimers of C/EBP-α and C/EBP-β [113]. In contrast, moderate constitutive levels of all C/EBP isoforms are present in untreated non-hepatic cells, i.e. rabbit lung fibroblasts and synoviocytes, but

their binding activities are not induced after treatment with IL-6. Instead, the rabbit *SAA2* promoter is activated by a novel, IL-6-responsive nuclear factor, SAF, which is present in the nuclei of a range of extrahepatic cells, including the above, as well as those of HepG2 cells. Mutational analyses have established that the rabbit *SAA2* promoter region has three SASs, all of which are essential for the IL-6-mediated transcriptional induction of the gene by SAF in non-hepatic cells [113]. The absence of C/EBP-binding activity in non-hepatic cells prompted the suggestion that C/EBP binding at extrahepatic sites is prevented by an inhibitor such as CHOP-10. This protein has strong homology to the bZIP DNA-binding domain of C/EBP family members through which it can form heterodimers with both C/EBP-α and C/EBP-β, thereby preventing them from binding their target sequences and activating gene transcription [175].

SAF cannot be detected until one hour after IL-6 treatment; this, together with the fact that SAF induction can be prevented by cycloheximide, indicates that this factor is made *de novo* in response to an IL-6-derived signal. Subsequent phosphorylation appears to be a requirement for SAF function, as its capacity to induce transcription can be reduced by phosphatase and protein kinase inhibitors and increased by protein phosphatase inhibitors [113].

At least three SAF cDNAs have been cloned by screening a rabbit brain cDNA expression library with a synthetic DNA probe containing multiple SASs [162]. All three belong to a protein family which share C-terminal zinc finger domains. SAF-1 appears to be the homolog of human MAZ/ZF87 and mouse Pur-1. The former is involved in the regulation of c-myc and serotonin 1 A receptor and the latter in insulin gene expression. SAF-5 and SAF-8 are unique. All three isoforms have similar binding sequence specificities upon interaction with the SAS element but they have different tissue expression profiles. An SAF-8-specific cDNA probe can detect approximately equal amounts of SAF mRNA in mouse heart, brain, spleen, lung, liver and kidney, testis and skeletal muscle, while SAF-5 is predominantly expressed in skeletal muscle, and a SAF-1 non-specific cDNA probe detects varying levels of SAF mRNA in all of the above tissues. The DNA-binding activity of SAF increases both in response to LPS or IL-6 and through post-translational phosphorylation modification [162].

Another zinc finger transcription factor, Sp1, is involved in the induction of the rabbit *SAA2* promoter in response to a range of mediators including LPS, minimally modified low-density lipoprotein (MM-LDL), IL-1 and IL-6 in extrahepatic cell lines [97,160,163,164]. The analysis of such cell lines may prove useful in understanding the potential role of A-SAA in the pathogenesis of disease. Human THP-1 cells express A-SAA and are useful for studying the impact of the acute-phase response on monocytes/macrophages, as they can be made to differentiate by incubation with LPS [96,99]. Such treatment of THP-1 cells, transfected with SAF cDNA, causes activation of the overexpressed SAF protein, which, in turn, transactivates a cotransfected SAS element–reporter gene construct [160]. Sp1 can interact with SAF by forming a stable heteromeric complex which has a higher affinity for the SAS element and is therefore a better transactivator of the *SAA2* promoter than either SAF or Sp1 alone [160]. MM-LDL can also induce SAF-binding and C/EBP-binding activity in THP-1 cells, thereby upregulating A-SAA mRNA expression, while native unmodified LDL has little effect [164]. As might be predicted from the above, mutations in the SAF and C/EBP, but not the NF-κB, recognition sequences reduce the responsiveness of the rabbit *SAA2* promoter to MM-LDL. Like LPS, MM-LDL treatment causes Sp1 and SAF to form a heteromeric complex with enhanced activity.

The activities of C/EBP family members are modified by treatment of THP-1 cells with MM-LDL [164]. Following such treatment, both the absolute amount and the activity of C/EBPα and C/EBPδ are increased, suggesting that there is *de novo* protein synthesis or import to the nucleus allied to functional enhancement. This contrasts with C/EBPβ and SAF which are subject to functional modulation without any apparent change in their concentration. As the pathogenesis of atherosclerosis involves changes in macrophage phenotypes possibly in response to oxidized lipoproteins, the above cellular processes may contribute to the expression of A-SAA in atherosclerotic lesions.

Cultured rabbit synovial fibroblasts, i.e. HI682 cells, have provided a useful model for studying A-SAA expression in the context of degenerative joint diseases such as rheumatoid arthritis. These cells produce A-SAA in response to inflammatory mediators by a mechanism involving SAF and Sp1 activation [163]. A-SAA is found in the synovial fluid of human rheumatoid arthritis patients [176,177], and it can induce collagenase [27,52] and the matrix metalloproteinases 2 and 3 [178] in both human and rabbit rheumatoid synovial fibroblasts. In addition, constitutive SAA3 mRNA expression has been demonstrated in rabbit corneal fibroblasts [179]. IL-1β and IL-6, which are present at high levels in patients with rheumatoid arthritis, both individually induce A-SAA gene expression in rabbit synovial cells, and in combination have a synergistic inductive effect [163]. This is achieved through the induction of the transcription factors Sp1 and certain SAF isoforms, distinct from constitutive SAF isoforms, in synovial cells. As in THP-1 cells, Sp1 forms a heteromeric complex with SAF to bind the *SAA2* promoter. Cytokine treatment causes Sp1 mRNA and protein levels to rise and mediates the post-translational modification of SAF; together these changes produce a net increase in DNA-binding activity. This process requires the cytokine-driven phosphorylation of Sp1 and, at least one of the SAF isoforms [163].

## Glucocorticoids

Naturally occurring glucocorticoids are steroid hormones produced by the adrenal cortex (Fig. 3) which affect intermediary metabolism (e.g. stimulation of glycogen deposition by the liver) and act as anti-inflammatory mediators (e.g. cortisone). Synthetic glucocorticoids such as dexamethasone, an analog of cortisol, are derivative steroid drugs and have proved to be potent suppressors of immune and inflammatory reactions; consequently they are widely used as therapeutic agents [180].

Glucocorticoid receptors (GRs) are part of an inactive cytoplasmic complex comprising heat shock proteins and immunophils [181,182]. When the GR is bound by glucocorticoids, it dissociates from the heat shock protein complex, and rapidly translocates to the nucleus where it acts as a composite transcription factor (reviewed in [183]). Glucocorticoids act via two main mechanisms. (a) The first is direct interaction with the promoters of genes, e.g. the IL-6 gene [184], that have a recognition site, the glucocorticoid-responsive element (GRE), to which the GR complex binds. GREs may be clustered with other transcription-factor-binding sites in so-called glucocorticoid-responsive units (GRUs) [185]. Although GRs are expressed in most cell types the specificity of cellular glucocorticoid responses are determined in part by the interaction of GRs with tissue-enriched transcription factors

at the GRUs. (b) The other mechanism is indirect interaction with the promoters of genes that do not have GREs or GRUs, via their effect on transcription factors such as AP-1 and NF-κB [186–188]. Glucocorticoids can physically interact with AP-1 to prevent its binding to recognition sites in target genes such as those encoding collagenase and IL-2, thereby repressing transcription [189–192]. Activated GR can also interact directly with NF-κB subunits (p65, p50 and c-Rel) thus preventing them from binding DNA. In addition, dexamethasone reduces the levels of p65 that can be achieved in the nucleus in response to TNF-α by either destabilizing the protein or, more likely, sequestering it in the cytoplasm [193]. Dexamethasone can also cause increased transcription from the gene encoding IκBα, the cytoplasmic inhibitor of NF-κB [194–196]. Consequently, even under conditions that promote IκBα degradation, increased synthesis of IκBα stimulated by glucocorticoids can be sufficient to bind NF-κB and retain it in the cytoplasm.

Glucocorticoids are upregulated by both IL-1α/β and TNF-α, and also enhance the capacity of these cytokines to induce APP synthesis in the liver. After their synthesis, glucocorticoids participate in the control of the acute-phase response by providing negative feedback on cytokine production (Fig. 3). Dexamethasone is a potent inhibitor of both IL-1β [197] and IL-6 [184], and acts at both a transcriptional and post-transcriptional level.

Dexamethasone enhances the induction of A-SAA mRNA by pro-inflammatory cytokines and LPS-conditioned medium in both human [100,106,108] and mouse [198] cell culture systems. Furthermore, increased levels of A-SAA are found in the serum of mice treated with dexamethasone, IL-1β and IL-6 [199]. Deletion reporter constructs containing 1.2 and 1.1 kb of the human *SAA2* and *SAA4* promoters, respectively, are unresponsive to dexamethasone (C. M. Uhlar, unpublished observation), indicating that the GREs or other control elements affected by glucocorticoids are more than 1 kb upstream of the transcription-start sites. There are precedents for the existence of eukaryotic enhancers, including GRUs, located many kilobases from the gene they regulate. For example, full glucocorticoid induction of rat tyrosine aminotransferase depends on the co-operative interaction of two GRUs 2.5 and 5.5 kb upstream of the transcription-start site [200,201].

## POST-TRANSCRIPTIONAL REGULATION OF A-SAA

Post-transcriptional mechanisms have also been implicated in the regulation of A-SAA. In early mouse studies, the 2000-fold LPS-mediated induction of mature hepatic A-SAA mRNA was not matched by the increase in the rate of transcription which, at 300-fold, was about an order of magnitude lower [46]. Furthermore, the absolute level of hepatic mRNA continued to accumulate even as the transcription rate was decreasing, i.e. the former peaked at 12 h whereas the latter peaked at 3 h. In a subsequent study, Saa3 mRNA levels in mouse liver-derived BNL cells stimulated with conditioned medium from mouse macrophage cells increased in the absence of any measurable effect on transcription [202]. Similar results have been obtained for human cells. In human Hep3B hepatoma cells treated with IL-1β plus IL-6, A-SAA transcription has been estimated to increase by only 23-fold during the time that mRNA accumulates to 1000 times normal levels [111]. In this study, the transcriptional peak was at 12 h whereas mRNA levels had not even begun to decline by 72 h. The disparity between transcriptional upregulation and the net effect on cytoplasmic mRNA concentration in these studies strongly suggests the involvement of post-transcriptional regulatory mechanisms such as increased mRNA stability.

Increased A-SAA mRNA synthesis does not inevitably result in proportionally increased A-SAA protein synthesis. In an early study, human PLC/PRF/5 hepatoma cells treated with monocyte-conditioned medium accumulated A-SAA mRNA [115]. From 3 h, the first time point at which it could be detected, A-SAA mRNA increased 76-fold by 24 h. Over the same time period, however, A-SAA protein synthesis only increased 4–6-fold, suggesting that the available pool of A-SAA mRNA was used efficiently early in the *in vitro* acute-phase response and progressively less efficiently over time. Similar results were obtained with IL-1β plus IL-6 treatment. The lower protein levels at later times do not appear to be due to either alterations in A-SAA protein export transit time or changes in the efficiency of translational initiation, but may be due to decreased A-SAA mRNA translational efficiency via a mechanism involving the slowing of ribosome migration [115].

The stability of a given mRNA and the efficiency with which it is translated may be dependent on poly(A) tail length [203–206]. In both mouse [207,208] and dog [21] models an acute-phase stimulus causes a rapid transcriptional upregulation of hepatic A-SAA which is closely followed by an increase in mRNA size because of a lengthened poly(A) tail. Although this hyperadenylation is not coincident with transcriptional modulation, it may be dependent on it or may be driven by the same underlying mechanism. A similar increase in poly(A) tail length is likely to occur in all mammals, including humans, after transcriptional upregulation *in vivo*; however, the first A-SAA mRNA that can be detected in cytokine-stimulated human hepatoma cells is the hyperadenylated form, thereby precluding a detailed analysis of the kinetics of, and relationships between, the above two processes *in vitro* [115]. After the appearance of 'full length' A-SAA mRNA *in vivo* or *in vitro*, a gradual reduction in A-SAA mRNA length, attributable to poly(A) tail shortening, has been observed in mouse, dog, human and arctic char [20,21, 115,207,208]. Moreover, in tissue culture studies using the transcriptional inhibitor actinomycin D, the intrinsic stability of A-SAA mRNA does not appear to be affected by poly(A) tail shortening (which is unaffected by treatment), suggesting that A-SAA mRNA degradation may require the *de novo* synthesis of a short-lived factor, e.g. a nuclease [111,115,209]. This speculation opens the intriguing possibility that cytokine induction may initiate a cascade of intracellular changes that control the post-transcriptional fate of A-SAA mRNA and the transcriptional products of other APP genes.

Deadenylation of A-SAA mRNA has been confirmed in a more recent study [210]. In unstimulated mice, the Saa1 poly(A) tail ranges from 20 to 30 adenines in size. However, during an azocasein-induced acute-phase response, this changes to 100–300 adenines after 8 h and to 20–70 adenines after 48 h. Trace amounts of decapped Saa1 mRNA can be detected in untreated, 8, 48 and 72 h samples, suggesting that decapping follows deadenylation and precedes degradation. This chronology is analogous to the situation in yeast where deadenylation at the 3′ end causes mRNA decapping at the 5′ end and subsequent 5′-3′ exonucleolysis (for references see [210]).

## SAA FUNCTION AND DISEASE ASSOCIATIONS

The A-SAA proteins have been highly conserved through evolution [18–20] and this, together with the dramatic

induction of A-SAA expression in response to potentially life-threatening physiological challenges, suggests a critical protective role in the acute-phase response. A-SAA may play a basic role in pathogen defense, as its mRNA levels rise continuously in response to increasing pathogen load in arctic char infected with the furunculosis-associated pathogen, *A. salmonicida*; in the final fatal stages of disease, A-SAA mRNA may constitute as much as 10% of total hepatic mRNA [20]. Moreover, other inflammatory mediators also elicit a dose-dependent response in other species, e.g. in Syrian hamsters increasing doses of IL-1, IL-6 and TNF drive the synthesis of increasing levels of hepatic A-SAA mRNA [211].

The best documented clinical condition associated with sustained high expression of A-SAA is amyloid A amyloidosis. However, the range of clinically important functions that have been proposed for the SAA family members include some that may also have other negative consequences during chronic inflammation. The functions fall into pro-inflammatory, lipid transport/metabolism related and anti-inflammatory categories; however, current research is placing the emphasis on the first two.

## Immune-related functions of A-SAA

There are two reported immune-related functions of A-SAA. First, A-SAA can induce ECM-degrading enzymes, such as collagenase, stromelysin, matrix metalloproteinases 2 and 3, which are important for repair processes after tissue damage [27,178,179]. However, prolonged expression of A-SAA, and the consequent long-term production of these enzymes, may play a role in degenerative diseases such as rheumatoid arthritis. Second, *in vitro* studies have provided compelling evidence that A-SAA can act as a chemoattractant for such immune cells as monocytes, polymorphonuclear leukocytes, mast cells and T lymphocytes [212–217]. If A-SAA has this property *in vivo*, its local production would result in the active recruitment of these cell types to inflammatory sites and the augmentation of local inflammation.

In addition to the capacity of A-SAA to induce ECM-degrading enzymes, two recent studies have reported induction of pro-inflammatory cytokines by A-SAA. In the first, rhSAA1.1 induced monocytic THP-1 cells to synthesize IL-1β, IL-1ra and soluble TNFR-II mRNA and protein but had no effect on the synthesis of soluble TNFR-I, IL-6 or TNF-α [215], while in the second, SAA–ECM complexes induced TNF-α secretion by human T lymphocytes [217]. The above suggest that A-SAA may, itself, have cytokine-like properties which would require cellular receptors or binding sites. Rabbit SAA3 can bind, both specifically and saturably to synovial fibroblasts, with an affinity of ≈ 20 nM and 800 000 binding sites per cell which is compatible with the existence of cellular receptors for SAA3 on these cells [218].

Chemoattractants, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMetLeuPhe) and the chemokines, act by binding seven-transmembrane-spanning G–protein-coupled receptors [219]. Several recent papers have presented evidence that A-SAA binds to such a receptor [216,220,221]. The hallmark of fMetLeuPhe and chemokine interactions with their receptors on monocytes via G–protein-dependent pathways are $Ca^{2+}$ mobilization and protein kinase C activation. A-SAA binding is characterized by some of the same phenotypic effects, i.e. protein kinase C appears to be involved in the A-SAA signaling pathways that result in monocyte recruitment, and, on binding, rhA-SAA transiently induces monocyte intracellular $Ca^{2+}$ levels from extracellular sources. Therefore it is probable that a G-protein is involved in A-SAA chemotaxis [220]. Furthermore, as the chemotactic effects mediated by rhA-SAA are pertussis toxin sensitive, they probably involve the $G_i$ subset of G-proteins [220]. Indeed the participation of a $G_i$-protein in A-SAA chemotaxis has subsequently been confirmed in another cell type, i.e. human mast cells which are normally resident in tissues but can accumulate at inflammatory sites [216].

Recently cross-desensitization experiments have established that the agonist fMetLeuPhe can bind to the same receptor as A-SAA [221]. Although rhA-SAA does not desensitize the $Ca^{2+}$ mobilization induced by chemokines or fMetLeuPhe, fMetLeuPhe can affect rhA-SAA-induced $Ca^{2+}$ mobilization; this suggests that A-SAA acts via a low-affinity fMetLeu Phe receptor. There are two seven-transmembrane-spanning G-protein-coupled receptors, FPR and FPRL1 (or lipoxin $A_4$ receptor), to which fMetLeuPhe can bind with high and low affinity, respectively, to effect $Ca^{2+}$ mobilization. The latter has also been reported to be a high-affinity receptor for the eicosanoid lipid metabolite, lipoxin $A_4$ and is expressed in monocytes, neutrophils and hepatocytes [222]. In human embryonic kidney cells transfected with and expressing FPRL1, but not in cells expressing FPR, rhA-SAA can induce both pertussis-toxin-sensitive $Ca^{2+}$ mobilization and migration. Furthermore, ligand-binding experiments show that rhA-SAA can specifically bind to such FPRL1-transfected cells ($K_d$ = 64 nM and 42 000 binding sites per cell), in addition to monocytes and neutrophils, and is a more efficient agonist of this receptor than fMetLeuPhe [221].

## Lipid-related functions of A-SAA

When A-SAA is released into the circulation it is incorporated into HDL [223,224], the class of lipoprotein particles that play an important role in the prevention of atherosclerosis by both mediating reverse cholesterol transport and inhibiting the lipid (LDL) oxidation that promotes foam cell formation [225,226]. Consequently, the association of A-SAA with HDL during acute inflammation may alter HDL metabolism and cholesterol transport and promote a pro-atherogenic phenotype [227–232]. There are two main hypotheses on the role that A-SAA plays in modulating cholesterol transport during inflammation (reviewed in [233]). One is that A-SAA alters reverse cholesterol transport to allow delivery of lipid, particularly cholesterol, via HDL to peripheral cells that may have an increased requirement for cholesterol to facilitate tissue regeneration at inflammatory sites [234,235]. The other is that A-SAA facilitates removal of the large quantities of cholesterol liberated at sites of tissue damage during inflammation [236,237].

In addition to its association with HDL, a number of lines of evidence implicate A-SAA in lipid metabolism/transport. A-SAA binds cholesterol and promotes its cellular uptake [238,239] and A-SAA-HDL particles have a higher affinity for macrophages and a lower affinity for hepatocytes than HDL [227,240]. Furthermore, several enzymes involved in cholesterol metabolism, including lecithin–cholesterol acyltransferase, group-IIA non-pancreatic secretory phospolipase $A_2$ ($sPLA_2$) and neutral cholesterol ester hydrolase, are affected by induction of A-SAA during the acute-phase response. Under normal physiologic conditions, ApoA-I is the main apolipoprotein of HDL (reviewed in [241]). However, during the acute-phase response, A-SAA becomes the major HDL-associated apolipoprotein and the particle becomes depleted of ApoA-I

**Table 2. Proteases involved in the degradation of A-SAA.**

| Proteases | References |
|---|---|
| Cell-associated activities | [290,291,292] |
| Serum serine proteases (thrombin, kallikrein and plasmin) | [293,266] |
| Elastase, collagenase and stromelysin | [293,295,296] |
| Cathepsin B | [296] |
| Aspartate proteases and cathepsin D | [297,298] |
| Cathepsin G | [264] |

[14]. As HDL is the site of cholesterol esterification which occurs through the action of lecithin–cholesterol acyltransferase, which, in turn, requires ApoA-I as an activating cofactor [242], the relative lack of ApoA-I on A-SAA–HDL during inflammation may account for the positive correlation observed between plasma A-SAA and unesterified cholesterol levels, and the negative correlation with lecithin–cholesterol acyltransferase activity [228]. sPLA2 is made by vascular smooth muscle cells [243] and is also found in human atherosclerotic lesions [244]. It was initially suggested that $sPLA_2$ would act on the HDL particle in a manner similar to that of hepatic lipase, hydrolyzing HDL phospholipids and redistributing cholesterol from the core to the surface, thereby facilitating its transfer to cell plasma membranes [245,246]. A recent study has found that human A-SAA–HDL is two- to threefold more susceptible to hydrolysis by $sPLA_2$ than is normal HDL [247]. Furthermore A-SAA, but not C-SAA, enhances the activity of $sPLA_2$ [246], and overexpression of $sPLA_2$ in transgenic mice can suppress HDL levels by 30% [234]. Consequently, the enhanced hydrolysis of acute-phase HDL by $sPLA_2$ appears to be mediated by A-SAA itself. Another product of $sPLA_2$ hydrolysis is arachidonic acid, which is the precursor of the pro-inflammatory eicosanoids. Human SAA1, but not apoA-I, can enhance the biosynthesis of the eicosanoids, thromboxane $A_2$ and prostaglandins $E_2$ and $F_{2\alpha}$ in calcium-ionophore-stimulated monocytes [76]. As atherogenesis may be promoted by the accumulation of released products of arachidonic acid, e.g. lysophosphatidylcholine which causes membrane damage, and toxic oxygenated fatty acids, A-SAA and $sPLA_2$ may be involved in the pathogenesis of this disease. Finally, neutral cholesterol ester hydrolase activity is increased in the presence of A-SAA favoring free cholesterol formation [237].

## Anti-inflammatory roles of A-SAA

Almost two decades ago, A-SAA was implicated in the suppression of *in vitro* immune responses to antigens by affecting T cell–macrophage interactions and helper T lymphocyte function [248–250]. Human A-SAA was subsequently found to be a potent inhibitor of lymphocyte, HeLa and MRC5 cell function [251]. A potential feedback relationship between SAA and immunoregulatory cytokines was proposed based on the observation that A-SAA inhibits IL-1-induced and TNF-induced fever in mice [252]. IL-1 and TNF cause fever by inducing prostaglandin $E_2$ synthesis in the hypothalamus; prostaglandin $E_2$ production correlates directly with the magnitude of the fever [253]. Platelet aggregation has also been reported to be inhibited by A-SAA [70,71], and A-SAA modestly induces prostaglandin $I_2$ which is also an antiaggregation agent [72]. As both platelets and the range of mediators released by them upon activation are involved in inflammatory and thrombotic processes, these findings suggest that A-SAA may act to down-regulate such pro-inflammatory events during the acute-phase response.

A-SAA has also been reported to bind to neutrophils and, like other apolipoproteins such as ApoA-I [254], inhibit the oxidative burst response, suggesting that it may help prevent oxidative tissue damage during inflammation [255,256]. However, this effect may be concentration-dependent; in a recent study, acute-phase concentrations of rhA-SAA could inhibit both directed neutrophil migration and degranulation [256], whereas the inhibition of respiratory burst was restricted to lower concentrations. These results suggest that A-SAA may produce quite different effects according to local concentration and that the anti-inflammatory effects intrinsic to this APP may be selective and specific rather than systemic.

## A-SAA, amyloid A protein and amyloidosis

A-SAA is the serum precursor of amyloid A protein [60,61] which is the principal component of the amyloid deposits found in the heterogeneous group of disorders, the amyloid A amyloidoses [257]. One of these, reactive amyloidosis, is a well-documented occasional clinical consequence of chronic inflammation (e.g. rheumatoid arthritis) and recurrent acute inflammatory episodes (e.g. tuberculosis).

The predominant amyloid A protein type found in amyloidotic tissues corresponds to the N-terminal two thirds of A-SAA, i.e. the first 76 residues of mature human A-SAA [60]. However, both smaller and larger amyloid A protein types, 45–95 residues, have also been found [10,62, 63,258,259]. Multiple proteolytic cleavage events may be involved in the processing of A-SAA as it appears to be degraded first into an intermediate product with the same size and antigenic properties as amyloid A protein and is subsequently processed further. Amyloid A amyloidosis may therefore be the result of the incomplete digestion, and consequent accumulation, of amyloidogenic intermediate peptides of A-SAA.

A large number of cell-associated and serum proteases have been implicated in the degradation of A-SAA (Table 2). A-SAA is probably degraded after its disassociation from HDL, as full-length A-SAA can be found in amyloid fibrils [260–263]. Furthermore, lipid-free A-SAA can be degraded [264] *in vitro* to form fibrils [265]. In addition, A-SAA degradation *in vivo* is inhibited by lipoproteins, in particular HDL [80,266], and differences between the plasma clearance rates of A-SAA and ApoA-I also suggest that the former is not associated with HDL when it is degraded [83,266].

Mouse A-SAA can bind to two of the major components of the basement membrane, i.e. laminin and type-IV collagen, with high and low affinity, respectively [267]. Its binding to laminin is inhibited by entactin, a protein that normally binds laminin. As the basement membrane matrix appears to be disrupted in the vicinity of amyloid deposits, these interactions further support the involvement of A-SAA as an active, rather than a passive, participant in the process of amyloidosis.

Inflammatory macrophages and reticuloendothelial cells have both been implicated in the formation of amyloid A protein and amyloid fibrils from A-SAA, each of which processes can occur intracellularly [268–271]. Mouse peritoneal macrophages can bind either HDL or A-SAA–HDL, which undergo receptor-mediated endocytosis and subsequent retro-endocytosis [272,273]. The binding of the latter probably involves heparin sulfate for which A-SAA has a binding site [78]. Mouse peritoneal macrophages can also endocytose

exogenous mouse Saa1 and Saa2, which are transported to endosome–lysosomes and are partially degraded to products similar in mass to amyloid A [274].

Although derivatives of SAA1 and SAA2 proteins are both found in human and mouse amyloid A deposits [275–277], those from SAA1 predominate [62,277–279]. This bias in favor of SAA1 deposition is supported by *in vitro* studies in which rhSAA1 had greater amyloid fibril-forming potential than either rhSAA2 or rhSAA4 [265].

## CONCLUSIONS

The highly conserved A-SAA proteins, which are all dramatically induced by various pro-inflammatory stimuli, have been identified in a wide range of vertebrate species. Their upregulation involves both transcriptional and post-transcriptional mechanisms. An intricate picture of the former is emerging with respect to cytokine-mediated induction and cell/tissue specificity. Many transcription factors that engage the A-SAA promoters have been identified, including members of the NF-κB and C/EBP families. These appear to be principally responsible for the synergistic induction of A-SAA observed in response to the IL-1-type, e.g. IL-1β and TNF-α, and the IL-6-type cytokines. Other transcription factors also regulate A-SAA expression; YY1 and AP-2 inhibit A-SAA gene transcription under various conditions, and SAF and Sp1 are involved in extrahepatic expression. It also appears that the kinetics of A-SAA gene induction in response to IL-1β and IL-6 is complex and depends critically on the order in which these mediators appear in the pro-inflammatory cytokine cascade.

The A-SAAs are highly conserved across evolutionarily distinct vertebrate species with respect to both their sequence and inductive capacity, and it is consequently generally assumed that they have a crucial, yet ill-defined, protective role during inflammation. The small size of A-SAA and the special circumstances under which it is induced make it likely that only one of the documented properties of A-SAA, i.e. immune or lipid transport/metabolism related, is that for which there is an over-riding need during the acute-phase response. Such a pre-eminent role would mandate the strong positive selection to which A-SAA has been subjected. The other properties attributed to A-SAA may merely reflect the consequences of the physiological adaptations made to facilitate its primary function. For example, the association of A-SAA with lipid, and the resulting impact on reverse cholesterol transport, may simply be due to the need to effectively sequester systemic pools of A-SAA before its targeted and/or timed release as an immune effector molecule. Future studies will more precisely define this and other aspects of A-SAA biology.

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CUTTING EDGE

# Cutting Edge: STAT Activation By IL-19, IL-20 and mda-7 Through IL-20 Receptor Complexes of Two Types[1]

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**IL-10-related cytokines include IL-20 and IL-22, which induce, respectively, keratinocyte proliferation and acute phase production by hepatocytes, as well as IL-19, melanoma differentiation-associated gene 7, and AK155, three cytokines for which no activity nor receptor complex has been described thus far. Here, we show that mda-7 and IL-19 bind to the previously described IL-20R complex, composed by cytokine receptor family 2–8/IL-20Rα and DIRS1/IL-20Rβ (type I IL-20R). In addition, mda-7 and IL-20, but not IL-19, bind to another receptor complex, composed by IL-22R and DIRS1/IL20Rβ (type II IL-20R). In both cases, binding of the ligands results in STAT3 phosphorylation and activation of a minimal promoter including STAT-binding sites. Taken together, these results demonstrate that: 1) IL-20 induces STAT activation through IL-20R complexes of two types; 2) mda-7 and IL-20 redundantly signal through both complexes; and 3) IL-19 signals only through the type I IL-20R complex.** *The Journal of Immunology*, **2001, 167: 3545–3549.**

Recently, a family of other cytokines with limited homology to IL-10 have been described (1). The first IL-10 homolog was called melanoma differentiation-associated gene 7 (mda-7)[3] because its expression was up-regulated during the in vitro differentiation of a melanoma cell line (2). Although this protein shows 22% amino acid identity with IL-10, it was not originally recognized as a secreted protein, and its biological activities remain poorly understood. The mouse ortholog of mda-7 was identified recently as a Th2-specific cytokine and called FISP, for IL-4-induced secreted protein (3). Its rat counterpart, called mob5, was suggested to play a role in ras oncogene-mediated neoplasia (4).

The *IL10* and *MDA7* genes have been mapped on chromosome 1q31–32, in a region where two additional IL-10-related genes, *IL19* and *IL20*, also were located. Little is known about IL-19, except that this gene is expressed by LPS-activated monocytes (5). The biological activities of IL-20 have been studied by using transgenic mice overexpressing this cytokine. These mice are characterized by neonatal lethality with skin abnormalities, including aberrant epidermal differentiation reminiscent of psoriasis lesions in human (6). An IL-20R complex was described as a heterodimer of two orphan class II cytokine receptor subunits: corticotropin-releasing factor (CRF) 2–8, proposed to be renamed IL-20Rα, and DIRS1, designated IL-20Rβ (6).

In addition to the chromosome 1q31–32 cluster, two other IL-10-related cytokines, AK155 and IL-22, are located on human chromosome 12q15, near the IFN-γ gene. AK155 is known to be up-regulated by *Herpes saimiri* infection of T lymphocytes, but its activity and receptor remain unknown (7). IL-22 was described originally as an IL-9-inducible gene and called IL-TIF, for IL-10-related T cell-derived inducible factor (8). IL-22 activities include induction of the acute phase response in hepatocytes and are mediated through a heterodimeric receptor composed of the CRF2–9/IL-22R subunit and the β-chain of IL-10R (9–11). In addition to its cellular receptor, IL-22 binds to a secreted member of the class II cytokine receptor family, which was called IL-22BP, and appears to act as a natural IL-22 antagonist (12, 13).

## Materials and Methods

### *Cell cultures and cytokines*

HT-29 intestinal epithelial cells were grown in IMDM medium supplemented with 10% FCS, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine. Human embryonic kidney (HEK) 293-EBV nuclear Ag cells were grown in DMEM medium supplemented with 10% FCS. IL-10 homologs were produced by transient expression in HEK293-EBNA cells by the Lipofectamine 2000 method (Life Technologies, Gent, Belgium). The coding sequences for mda-7, IL-19, and IL-22 were amplified by RT-PCR from RNA of T cells stimulated with anti CD3 Ab. The IL-20 coding sequence was amplified from skin RNA. These cDNAs were cloned into pCEP4 plasmid (Invitrogen, Groningen, the Netherlands) under the control of the CMV promoter. mda-7-Flag, IL-19-flag, IL-20-flag and IL-22-flag were generated from the pCEP4-cytokine constructs by mutating the STOP codon and introducing a sequence encoding a C-terminal flag: Gly-Gly-Gly-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. The IL-22BP-Ig fusion cDNA was produced as described before (12). For Western blot analysis, 10 μl of HEK293 supernatant was mixed with Laemmli sample buffer and boiled for 5 min before SDS-PAGE and transfer onto a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). The membrane

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[3] Abbreviations used in this paper: mda-7, melanoma differentiation-associated gene 7; CRF, cytokine receptor family; HEK, human embryonic kidney.



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was probed with biotinylated anti-flag Ab (25 μg/ml) and with streptavidin-HRP (1/5000; Amersham). An ECL detection kit (Amersham) was used for expression of chemiluminescence. The chemiluminescence signal was detected and quantified with a Kodak (Rochester, NY) Digital Science Image Station 440CF. Anti-phospho-STAT3 Western blots were performed as described previously (8).

The DIRS1/IL-20Rβ cDNA was amplified by RT-PCR from K562 leukemia cells and cloned into pCEP4 plasmid. The IL-22R cDNA was amplified by RT-PCR from the HepG2 hepatoma cell line before cloning into the pEF-BOSpuro expression vector (14). The CRF2–8/IL-20Rα cDNA was amplified by PCR from a human placenta cDNA library (Clontech Laboratories, Palo Alto, CA), and cloned into the pCDEF3 plasmid. Anti-IL-10Rβ and anti-flag Abs were purchased from Peprotech (London, U.K.) and from Sigma (Bornem, Belgium), respectively. To produce anti-hIL-22R Abs, we transfected P815 mastocytoma cells with the hIL-22R cDNA in the pEF-BOS plasmid before injection into DBA/2 mice. After rejection of the tumors, the sera of these mice had high titers of neutralizing anti-hIL-22R Abs and were used at a 1/500 dilution.

### *Luciferase assays*

The cytokine response was assessed by measuring luciferase production by cells transfected with the pGRR5 construct, (provided by Dr P. Brennan, Imperial Cancer Research Fund, London, U.K.). This construct contains five copies of the STAT-binding site of the FcγRI gene inserted upstream from a luciferase gene controlled by the TK promoter. Transfections of HT29 and HEK293 cells were performed as follows.

HT-29 cells were electroporated ($10^7$ cells in 400 μl, 250 V, 192 Ω, 1200 μF) with 15 μg of pGRR5 and 15 μg of each receptor cDNA, separately or in combination. Transfected cells were seeded in 96-well plates, incubated for 5 h at 37°C, and then preincubated, or not, for 1 h with anti-IL-22R antiserum (1/500) or with anti-IL-10Rβ Abs (6 μg/ml). Next, the cells were stimulated with each cytokine for 2 h. Luciferase activity was measured with the Luclite plus Assay System kit (Canberra-Packard, Meriden, CT) with a Top Count microplate scintillation counter (Canberra-Packard).

HEK293-EBNA cells were seeded in 24-well plates (Nunc, Roskilde, Denmark) for 24 h. Transfections were conducted by using the Lipofectamine method (Life Technologies, Gent, Belgium), with 500 ng of plasmid encoding IL-22R, IL-20Rβ, or IL-20Rα and with 100 ng of pGRR5. As an internal control, we used 100 ng of pRL-TK vector (Promega, Madison, WI) containing the *Renilla* luciferase gene under the control of the TK promoter. After 20 h, transfected cells were stimulated with cytokines, and 2 h later, cells were pelleted and lysed. Luciferase activity was monitored with the Dual-Luciferase Reporter Assay System kit (Promega).

### *IL-22BP interaction assays*

Specific interactions between IL-22BP and cytokine-flag fusion proteins were assessed directly or indirectly by ELISA, as follows. Reacti-Bind Maleic Anhydride Activated Polystyrene plates (Pierce, Rockford, IL) were coated overnight at 4°C with 12.5 μg/ml of anti-flag Ab in PBS. The plates were incubated 2 h at 37°C with 50 μl of cytokine-flag fusion proteins (HEK293 supernatants). A total of 10% of supernatant of IL-22BP-Ig was added for 2 h, and bound IL-22BP-Ig was detected by using anti-mouse IgG3 polyclonal Abs coupled to peroxidase (Southern Biotechnology Associates, Birmingham, AL). The enzymatic activity was measured as described previously (12). In the indirect assay, we tested the inhibitory effect of IL-10 homologs on the binding of IL-22BP to IL-22. For this purpose, IL-22BP-Ig (10%) was preincubated with IL-10 homologs 2 h before incubation with Reacti-Bind plates (Pierce) that had been coated with rIL-22 as described previously (12).

## Results

### *STAT activation by IL-10 homologs in class II cytokine receptor-transfected cells*

To characterize the interactions between IL-10 homologs and receptors belonging to the class II cytokine receptor family, we expressed mda-7, IL-19, IL-20, and IL-22 as fusion proteins with a C-terminal flag sequence by transient transfection of HEK293 cells. Protein production was checked by Western blot with an Ab specific for the flag peptide (Fig. 1*A*). HEK293 cells secreted mda-7, IL-19, and IL-22 proteins with a heterogeneous MW of 23–30 kDa, most likely resulting from glycosylation. The IL-20-flag protein is secreted as a single band with a size of ~18 kDa, suggesting that this cytokine is not glycosylated. Quantification of the chemiluminescence signal indicated that IL-19 and IL-22 were produced at a similar level, whereas IL-20 and mda-7 were produced 7-fold less.

These HEK293 supernatants were used to assess the interaction with class II cytokine receptors. A first set of experiments were performed in HT-29 cells, which endogenously express IL-22R and IL-10Rβ. STAT activation induced by IL-22 was monitored with the pGRR5 luciferase reporter (9). As shown in Fig. 1*B* (*top left*), these cells failed to respond to the other IL-10 homologs. When HT-29 cells were transfected with the IL-20Rβ cDNA, both mda-7 and IL-20 induced luciferase production. Interestingly, this effect was completely blocked by an anti-IL-22R antiserum, suggesting that mda-7 and IL-20 can activate STAT factors through a new IL-20R complex composed by IL-22R and IL-20Rβ (Fig. 1*B*, *bottom left*).

When cells were transfected with both IL-20Rα and IL-20Rβ cDNAs, they became responsive to mda-7, IL-20, and IL-19, and the luciferase production was not affected anymore by anti-IL-22R

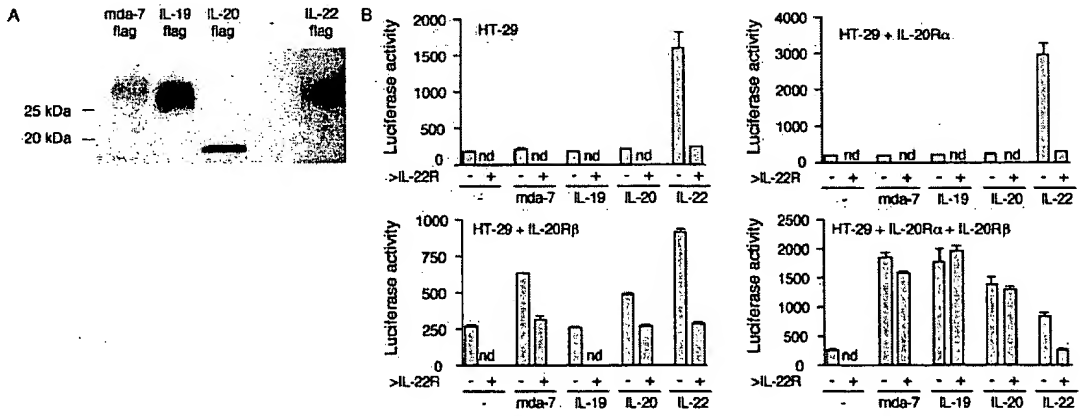


FIGURE 1. Human IL-10 homolog production and activity. *A*, The cDNAs encoding mda-7, IL-19, IL-20, and IL-22 tagged with a flag sequence were transfected in HEK293 cells. After 4 days, supernatants were analyzed by Western blotting with an Ab raised against the flag peptide. *B*, HT-29 cells were transfected with the pGRR5 luciferase construct with or without IL-20Rα and IL-20Rβ as indicated. Cells were preincubated 1 h with or without anti-IL-22R antiserum (1/500) before stimulation with 1% of HEK293 supernatants. Luciferase activity was monitored 2 h later and is expressed in arbitrary units.

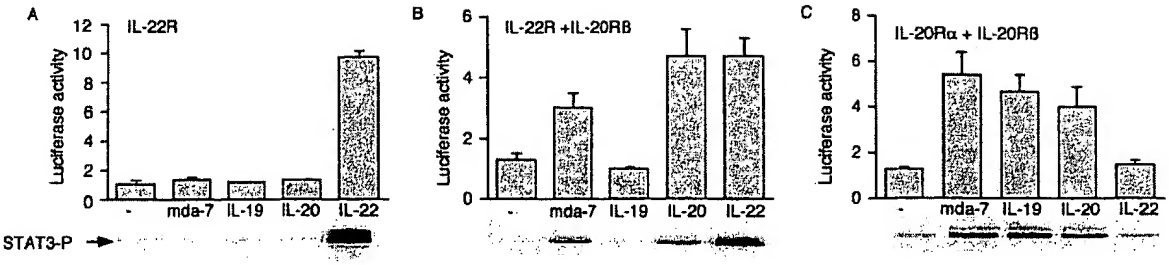


**FIGURE 2.** Activity of IL-10 homologs in HEK293 cells. HEK293 cells were transfected with the pGRR5 luciferase construct and cDNAs encoding IL-22R (*A*), IL-22R and IL-20Rβ (*B*), or IL-20Rα and IL-20Rβ (*C*). Cells were stimulated with 10% of mda-7, IL-19, IL-20, or IL-22 supernatant. Luciferase activity was monitored 2 h later. The results are normalized by using *Renilla* luciferase as an internal control. Western blot analysis for STAT3 phosphorylation was performed after 15 min of stimulation.

Abs (Fig. 1*B*, *bottom right*), indicating that this activity was independent from this chain. Finally, on transfection with the IL-20Rα cDNA alone, we failed to detect any response to mda-7, IL-19, and IL-20 (Fig. 1*B*, *top right*), confirming that IL-20Rβ is required for this process.

To characterize further the different types of receptor complexes, we used HEK293 cells, which express endogenous IL-10Rβ but not IL-22R. Untransfected HEK293 cells did not respond to any IL-10 homolog (data not shown). When the IL-22R cDNA was transfected, only IL-22 induced luciferase production and STAT-3 phosphorylation (Fig. 2*A*). Cells transfected with IL-22R and IL-20Rβ responded not only to IL-22 but also to IL-20 and mda-7 (Fig. 2*B*), whereas IL-20Rβ alone did not confer any cytokine responsiveness (data not shown). Transfection of both IL-20Rα and IL-20Rβ cDNAs allowed for STAT activation by mda-7, IL-19, and IL-20, but not IL-22 (Fig. 2*C*). No response was observed in cells transfected with the IL-20Rα cDNA alone (data not shown). In all cases, luciferase induction correlated with phosphorylation of STAT-3, as analyzed by Western blotting (Fig. 2). Similar results were obtained with HEK293 supernatants containing the wild-type cytokines.

### *Comparison of IL-20 and mda-7 sensitivity conferred by both types of IL-20R complexes*

The observation that two different receptor complexes allowed for the response to IL-20 and mda-7 raised the possibility that each complex would respond preferentially to one cytokine. To test this hypothesis, we analyzed the response of HT-29 cells, transfected either with IL-20Rβ alone or both IL-20Rα and IL-20Rβ, to different dilutions of mda-7, IL-19, and IL-20 supernatants. When both IL-20Rα and IL-20Rβ were transfected, mda-7 and IL-20 dilutions showed a similar dose-response curve, indicating a similar sensitivity to both cytokines (Fig. 3, *bottom*). The activity of IL-19, but not those of mda-7 and IL-20, could be detected with 0.1% of supernatant, in agreement with the higher concentration of IL-19 supernatants. When only IL-20Rβ was transfected, HT-29 cells showed a better response to mda-7 at nonsaturating dilutions (1% and 0.1% supernatant), indicating that this type of complex is more sensitive to mda-7 (Fig. 3 *top*). Similar results were obtained in HEK293 cells (data not shown).

### *IL-20Rβ cannot substitute for IL-10Rβ in IL-22 signaling*

The finding that IL-22R can associate not only with IL-10Rβ as described previously, but also with IL-20Rβ raised the possibility that the complex of IL-20Rβ with IL-22R could mediate an IL-22 response. Because IL-10Rβ is ubiquitously expressed, we could not address this question by direct transfection, but the role of IL-10Rβ was assessed with an anti-IL-10Rβ Ab. As shown in Fig. 4, this Ab could block the IL-22 activity both in control HT-29 cells and in cells transfected with the IL-20Rβ cDNA, indicating that IL-20Rβ cannot substitute for IL-10Rβ when the latter chain is not accessible to IL-22. The same Ab did not affect the activity of mda-7 or IL-20 in the same cells (data not shown).

### *mda-7, IL-19, and IL-20 do not bind IL-22BP*

IL-22BP has been shown to bind IL-22 (12, 13), but nothing is known concerning its ability to bind other IL-10 homologs. The fact that this soluble receptor exhibits the same degree of homology with the extracellular domains of IL-22R and IL-20Rα prompted us to test the hypothesis that IL-22BP could also bind IL-20. In a first set of experiments, we tested the ability of the IL-10 homologs to compete for the binding of IL-22BP to insolubilized IL-22. Microtiter plates were coated with rIL-22 and incubated with an IL-22BP-Ig fusion protein in the presence of IL-10 homologs. The interaction between IL-22 and IL-22BP was detected with an anti-Ig Ab. As shown in Fig. 5*A*, only IL-22 supernatants were able to block IL-22BP binding. To directly assay the

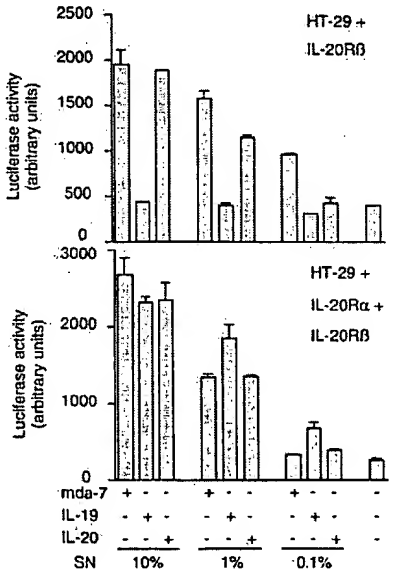


**FIGURE 3.** Comparison of IL-20 and mda-7 sensitivity with both types of IL-20R complexes. HT-29 cells were transfected with the pGRR5 luciferase construct and the cDNAs encoding IL-20Rβ alone (*top*), or IL-20Rα and IL-20Rβ (*bottom*). Cells were stimulated with different dilutions of mda-7, IL-19, and IL-20 supernatants for 2 h before measuring luciferase activity.

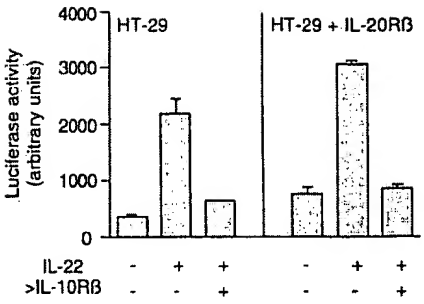


**FIGURE 4.** IL-20R$\beta$ cannot substitute for IL-10R$\beta$ in IL-22 signaling. HT-29 cells were transfected with the pGRR5 luciferase construct and the IL-20R$\alpha$ cDNA. Cells were preincubated 1 h with anti-IL-10R$\beta$ Ab before stimulation with 10% supernatant from IL-22- or mock-transfected HEK293. Luciferase activity was monitored 2 h later.

interaction between IL-10 homologs and IL-22BP, we coated microtiter plates with anti-flag Ab before incubation with flag-tagged IL-10 homologs. IL-22BP-Ig was added, and interaction was checked with an anti-Ig Ab. As shown in Fig. 5*B*, only IL-22 was able to bind IL-22BP-Ig, and no other IL-10 homolog showed the same activity.

## Discussion

Sharing receptor subunits is a well-known feature within class I cytokine receptors and has allowed to define subfamilies based on the involvement of subunits such as $\beta$c, gp130, and IL-2R$\gamma$. Among the class II cytokine receptors, the only example of a shared receptor so far was the IL-10R$\beta$ chain, which is involved in both IL-10 and IL-22 signaling (9–11). In this paper, we show that IL-22R and DIRS1/IL-20R$\beta$ are also shared by different receptor

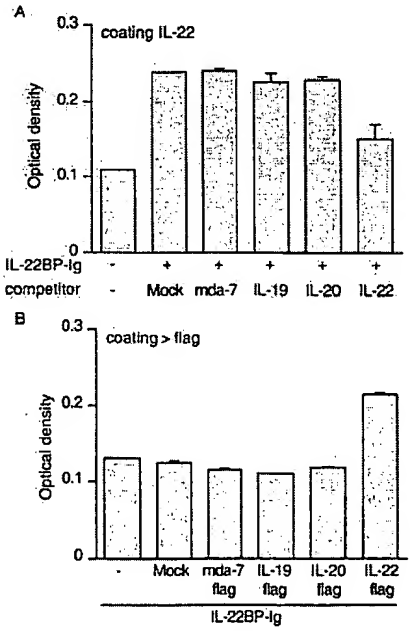


**FIGURE 5.** IL-22BP interacts specifically with IL-22. *A*, Plates were coated with rIL-22 before addition of IL-22BP-Ig preincubated with IL-10 homologs. Specific interactions were detected with rabbit polyclonal anti-Ig Abs. *B*, Plates were coated with anti-flag Ab. Supernatants containing flag-tagged IL-10 homologs were added before incubation with IL-22BP-Ig. Specific interactions were detected with a rabbit polyclonal anti-mouse Ig.

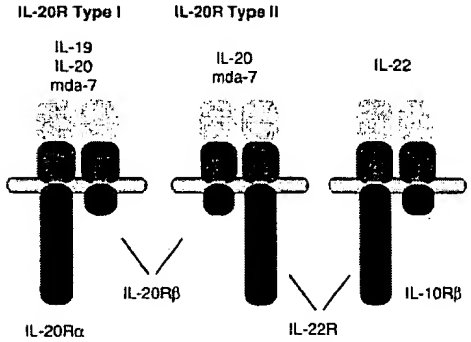


**FIGURE 6.** Schematic representation of IL-22R and IL-20R complexes.

complexes. The IL-20R$\beta$ subunit can associate either with IL-20R$\alpha$, leading to a functional receptor for IL-19, IL-20, and mda-7 (type I IL-20R complex). IL-20R$\beta$ also can associate with the IL-22R subunit and lead to a functional receptor for IL-20 and mda-7, but not for IL-19 (type II IL-20R complex), as schematically represented in Fig. 6. Additional experiments are needed to determine which of these chains serve as an actual ligand binding component or as a Jak-recruiting subunit. Alternatively, these receptor subunits may be expressed as preassociated complexes at the surface of the cells.

IL-20-transgenic mice show neonatal lethality and skin abnormalities, including thickened epidermis and expression of markers of hyperproliferation (6). Our observations strongly suggest that IL-19 and mda-7 can have a similar activity. Interestingly, IL-19 acts only on type I IL-20R and should therefore recapitulate only partly IL-20 activities. By contrast, IL-20 and mda-7 seem to behave similarly regarding both complexes. Noticeably, expression of the rat ortholog of mda-7 seems to be up-regulated during wound healing, a process that definitely involves keratinocyte proliferation (15).

Although mda-7 was originally identified several years ago (2), its activities and mode of action remain poorly understood. This protein was reportedly expressed intracellularly and was shown to induce apoptosis in certain tumor cell lines by an unknown mechanism (16, 17). On transfection of the mda-7 cDNA in HEK293 cells, we found most of the protein in the supernatant, indicating that it can be secreted, at least in this cell type. Secretion of the rat and mouse orthologs of mda-7 in various cell types also has been reported (3, 4). Together with our observation that exogenous mda-7 binds to the IL-20R complexes, these data support the hypothesis that mda-7 acts as a paracrine or autocrine factor. However, it remains possible that mda-7 might be expressed either as a cytoplasmic protein, inducing cell growth inhibition and apoptosis, or as a secreted protein acting on various cell types through IL-20R complexes.

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# Interleukins 19, 20, and 24 Signal through Two Distinct Receptor Complexes

## DIFFERENCES IN RECEPTOR-LIGAND INTERACTIONS MEDIATE UNIQUE BIOLOGICAL FUNCTIONS*

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**Cytokines that signal through Class II receptors form a distinct family that includes the interferons and interleukin 10 (IL-10). Recent identification of several IL-10 homologs has defined a cytokine subfamily that includes AK155, IL-19, IL-20, IL-22, and IL-24. Within this subfamily, IL-19, IL-20, and IL-24 exhibit substantial sharing of receptor complexes; all three are capable of signaling through IL-20RA/IL-20RB, and IL-20 and IL-24 both can also use IL-22R/IL-20RB. However, the biological effects of these three cytokines appear quite distinct: immune activity with IL-19, skin biology with IL-20, and tumor apoptosis with IL-24. To more fully elucidate their interactions with the receptor complexes, we have performed a series of *in vitro* assays. Reporter, proliferation, and direct STAT activation assays using cell lines expressing transfected receptors revealed differences between the receptor complexes. IL-19 and IL-24 also exhibited growth inhibition on a cell line endogenously expressing all three receptor subunits, an effect that was seen at cytokine levels two orders of magnitude above those required for STAT activation or proliferation. These results demonstrate that, although this subclass exhibits receptor complex redundancy, there are differences in ligand/receptor interactions and in signal transduction that may lead to specificity and a distinct biology for each cytokine.**

Interleukin 10 (IL-10)[1] and the related cytokines IL-20 (1), IL-19 (2), IL-24 (mda-7) (3), IL-22 (4, 5), and AK155 (6) form a distinct subfamily of ligands that bind and signal through Class II cytokine receptors. The IL-10 receptor (for review, see Ref. 7) consists of two subunits, a private alpha subunit (IL-10RA) and a beta subunit (IL-10RB), previously known as CRF2–4, that is also part of the IL-22 receptor complex (4). IL-10 modulates gene expression in responsive cell types through activation of the Jak/STAT signal transduction pathway (for reviews, see Refs. 8–11), in particular activating STATs 1, 3, and 5 (7, 12).

Recently, extensive cross-reactivity of the IL-10 subfamily ligands IL-20, IL-19, and IL-24 with two different receptor complexes consisting of IL-20RA/IL-20RB and also IL-22R/IL-20RB was observed (13, 14).[2] Although activation of a single receptor complex by multiple ligands is not unusual (*e.g.* the interferon $\alpha/\beta$ system), sharing of single receptor subunits between distinct ligand-specific complexes is more common. The sharing of identical receptor complexes within the IL-10 ligand subfamily raises the question of signal specificity. Each of the three ligands we have studied, IL-20, IL-19, and IL-24, appears to have unique biological activity. IL-20 has shown *in vitro* activity on keratinocytes, and the IL-20 transgenic phenotype has led to further investigation of IL-20 in skin biology (1). IL-19 has been reported to directly affect immune cells (2). Mice transgenic for IL-19, with the same promoters and similar expression levels as with IL-20, have no overt skin phenotype.[3] IL-24 appears to function as a proapoptotic cytokine in a variety of tumors (15–18). These observations suggest distinct physiological roles for each ligand despite the sharing of receptor complexes. We therefore designed a series of experiments to better elucidate the interactions between these three ligands and their receptor complexes.

Here we show that all three receptor subunits are expressed in similar cell types in lung tissue, suggesting that at least in some instances a given cell type may respond to a given ligand through both receptor complexes simultaneously. To more fully study the interactions of each ligand with each receptor complex, we have performed both proliferation and reporter assays in two cell types stably transfected with each complex. The results of these assays indicate that each receptor complex responds to its respective ligands in a quantitatively similar fashion. However, growth inhibition experiments on the ovarian carcinoma cell line NIH:OVCAR-3, which endogenously expresses all three receptor subunits, suggest both that receptor recognition alone cannot adequately explain differences in ligand activity and that alternative signaling pathways may be involved. Another series of experiments to further study the activation of individual STAT proteins showed that in transfected cells STAT3 is activated at low (physiological) ligand concentrations, whereas STAT1 activation is seen at much higher levels.

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[1] The abbreviations used are: IL-10, interleukin 10; STAT, signal transducers and activators of transcription; RT, reverse transcription; wt, wild type; SR, soluble receptor; BHK, baby hamster kidney cells.

[2] J. Parrish-Novak, W. Xu, T. Brender, L. Yao, C. Jones, J. West, C. Brandt, L. Jelinek, K. Madden, P. A. McKernan, D. C. Foster, S. Jaspers, and Y. A. Chandrasekher, unpublished data.

[3] Transgenic group, ZymoGenetics, unpublished observations.

## EXPERIMENTAL PROCEDURES

*RT-PCR Analysis on Human Tissues*—RT-PCR was performed on a human Rapid-Scan gene expression panel (Origene Technologies, Inc.) using primers 5′-ccccagacacggtctacagcat-3′ and 5′-gggtcaggccgaagaactcatat-3′ to amplify a 440-bp fragment of human *IL22R*. PCR conditions are 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 72 °C for 90 s, then a final extension step of 72 °C for 2 min. (See www.origene.com/ge_rs_dlink.html for a description of Rapid-Scan panel construction and control message amplification.)

*In Situ Hybridization*—Hybridizations were carried out as described previously for IL-20 receptor subunits (1). For *IL22R*, two independent probes were designed and corresponded to nt 1910–2783 and nt 208–1081 with respect to sequence AF286095. BLASTn searches using our databases, which include all known class II cytokine receptors, confirmed that the probes were specific. A human alpha actin probe was used as a positive control for the tissue samples, which corresponds to nt 603–1328 of sequence NM_001613. A sense probe was used as a negative control. PCR products containing the working sequence of the T7 RNA polymerase promoter were used as templates for synthesis of digoxigenin-labeled antisense RNA probes (Riboprobe *in vitro* Transcription System, Promega). Hybridization was carried out at 60 °C with 50% formamide/2× SSC. The signals were amplified with two to three rounds of tyramide signal amplification (TSA *in situ* indirect kit, PerkinElmer Life Sciences) and visualized with Vector Red substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin. Four different samples of normal human lung tissue were used with each probe. All tissues were tested with positive control probes and confirmed to be suitable for *in situ* hybridization analysis.

*Luciferase Assay*—Luciferase reporter assays were performed as described previously (19) using BHK570 cells stably transfected with IL-20RA and IL-20RB or with IL-22R and IL-20RB and utilizing the STAT-driven luciferase reporter cassette. The STAT elements included in this construct are STATs 1, 3, 4, 5, and 6. Cells were switched to serum-free medium overnight prior to treatment with serial dilutions of IL-19, IL-20, and IL-24 in the presence or absence of IL-20RA/IL-20RB-soluble receptor. Cells were lysed and luciferase reporter activity was determined in triplicate for each data point using a Berthold Micro-Lumat Plus luminometer.

*BaF3 Proliferation Assays*—The BaF3 cells were stably transfected with full-length Class II receptor subunits alone or in combinations and treated with IL-20, IL-19, and IL-24. For end point proliferation assays, cells were cultured at 5000 cells/well with variable cytokine concentrations for 72 h at 37 °C. Alamar Blue (Accumed) was added to the cells, and plates were read 24 h later on a *f*max plate reader (Molecular Devices, Sunnyvale, CA) using the Softmax Pro program, 544-nm excitation, and 590-nm emission. For kinetic proliferation assays, cells were cultured at 5000 cells/well with 60 pM human IL-20, IL-19, or IL-24 in 96-well flat-bottomed plates at 37 °C. Each well was pulsed with 250 nCi of [$^3$H]thymidine 6 h prior to harvest. Plates were harvested and counted at 24, 48, 72, and 96 h of culture.

*Growth Inhibition Assay*—The human ovarian carcinoma cell line NIH:OVCAR-3 (20) was obtained from ATCC and cultured in Invitrogen RPMI 1640 medium with L-glutamine supplemented with 20% fetal bovine serum, 1% sodium pyruvate, 20 mM Hepes, and 10 μg/ml insulin. For growth inhibition assays, cells were plated in culture media at 5000 cells/well in 96-well flat-bottomed tissue culture-treated plates (Corning Costar) and allowed to adhere for 24 h in a 37 °C, 5% $CO_2$ incubator. Cells were treated in triplicate with each dose of each cytokine for 48 h. Relative live cell counts were determined using MTT Cell Titer 96 Nonradioactive Proliferation assay (Promega) according to the manufacturer's specifications. Percent inhibition was defined as the average of $100 - 100 \times [(A_{572} - A_{650}$ for unknown)/$(A_{572} - A_{650}$ for growth media control)].

*STAT Translocation Assay*—BHK570 cells stably expressing each receptor complex were plated in flat-bottomed 96-well plates at 2000 cells per well. The next day, cells were refed with serum-free medium and starved for 5–16 h. Cytokines were serially diluted into serum-free medium then added to cells to achieve final cytokine concentrations of 0.15–20 nM. Plates were incubated at 37 °C for 45 min (STAT1) or 20 min (STAT3). Immediately following incubation, plates were washed, fixed, and stained using protocols provided with STAT1 and STAT3 HitKit reagent kits (Cellomics, Inc., Pittsburgh, PA). Plates were analyzed using the ArrayScan II instrument running the nuclear translocation protocol (Cellomics, Inc.). A minimum of 100 cells per well was analyzed. Data were subjected to one-tailed unpaired *t* test with Welch's correction using Prism (GraphPad) software.

*Protein Expression*—Untagged recombinant human IL-20 was produced in baculovirus. C-terminally FLAG-tagged IL-19 and IL-24 were expressed in BHK570 cells. IL-20RA- and IL-20RB-soluble receptors were expressed as homodimeric IgG fusion proteins in BHK570 cells. For construction of heterodimeric IL-20RA/IL-20RB, the extracellular domains of IL-20RA and IL-20RB were fused to human IgG with a Gly-Ser (×4) spacer between. In addition, the IL-20RA carried a C-terminal EE affinity tag (GEYMPME), and the IL-20RB carried a C-terminal $His_6$ tag. The receptor subunits were co-expressed in BHK-570 cells, and the IgG fusions were isolated from the culture media by protein-A chromatography. The heterodimers were purified away from the homodimers by immobilized metal chromatography utilizing an imidazole step gradient elution.

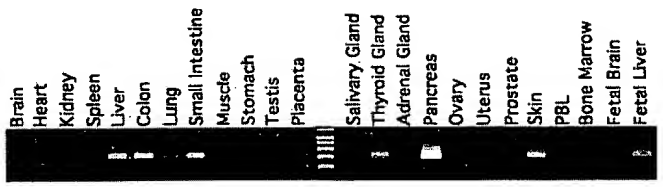



FIG. 1. **RT-PCR analysis of *IL22R*.** PCR was performed on a Rapid-Scan human tissue panel (Origene) according to the manufacturer's instructions. Shown is the gel representing the 100× (100 pg each cDNA) series.

## RESULTS

*Expression of Receptor Subunits*—Recent studies in our laboratory and others have shown redundant interactions between the IL-10 homologs IL-19, IL-20, and IL-24 and their newly identified receptor complexes IL-20RA/IL-20RB and IL-22R/IL-20RB (1, 13, 14). To better understand the functional relevance of the receptor redundancy found in this IL- 20 subfamily, we performed a variety of experiments. Expression analysis using RT-PCR showed that *IL20RA* is the most widely expressed of the three receptor subunits. Previous RT-PCR analysis using Origene panels showed that both *IL20RA* and *IL20RB* mRNAs are highly expressed in skin and testis, and are also expressed in a variety of other tissues, including the lung and ovary (1). Because IL-22R is a shared alpha subunit, we evaluated an identical Origene panel for the expression of *IL22R* mRNA (Fig. 1). Table I provides a summary of our previous data directly compared with the new data shown in Fig. 1. *IL22R* is expressed in a few tissues that lack *IL20RB* expression, notably pancreas, small intestine, and fetal liver. With the exception of peripheral blood lymphocytes, *IL20RB* was only expressed in tissues that also showed *IL20RA* expression; most of these lacked *IL22R* expression. Co-expression of *IL22R* and *IL20RB* was only observed in tissues that also expressed *IL20RA*. Of these tissues, skin and lung exhibited robust expression of all three receptors.

Because *IL20RA*, *IL20RB*, and *IL22R* are all expressed in the lung, we performed *in situ* hybridization on lung sections to evaluate whether the same cell types express all three receptors. The results show that epithelial cells as well as immune infiltrates exhibit positive staining for all three receptor subunits (Fig. 2). Taken together, the RT-PCR and *in situ* hybridization analyses show that the cellular/tissue content can be similar for *IL20RA*, *IL20RB*, and *IL22R*.

*STAT Reporter Activation*—To evaluate functional interactions between the IL-20 ligand subfamily and the two receptor complexes, we utilized cells with stably transfected receptors and those determined to express the receptor subunits endogenously and assayed a variety of endpoints. In the first of these experiments, BHK570 cells were stably transfected with IL-20RA/IL-20RB, IL-22R/IL-20RB, or individual receptor subunits alone and treated with increasing amounts of IL-19, IL-20, and IL-24. These cells were also stably transfected with a reporter construct consisting of the firefly luciferase gene driven by promoter/enhancer sequences comprised of tandem

TABLE I
*Summary of RT-PCR expression analysis of IL20RA, IL20RB, and IL22R in human tissues*

| | *IL20RA* | *IL20RB* | *IL22R* |
|---|---|---|---|
| Brain | + | − | − |
| Heart | ++ | +/− | − |
| Kidney | +/− | − | +/− |
| Spleen | − | − | − |
| Liver | +/− | − | + |
| Colon | +/− | − | + |
| Lung | + | + | + |
| Small intestine | +/− | − | + |
| Muscle | +/− | + | − |
| Stomach | + | − | − |
| Testis | ++ | ++ | − |
| Placenta | + | + | − |
| Salivary gland | + | − | − |
| Thyroid gland | + | − | + |
| Adrenal gland | +/− | +/− | +/− |
| Pancreas | + | − | ++ |
| Ovary | + | + | − |
| Uterus | + | − | − |
| Prostate | ++ | − | − |
| Skin | ++ | ++ | + |
| PBL | − | +/− | − |
| Bone marrow | − | − | − |
| Fetal brain | − | − | − |
| Fetal liver | − | − | + |

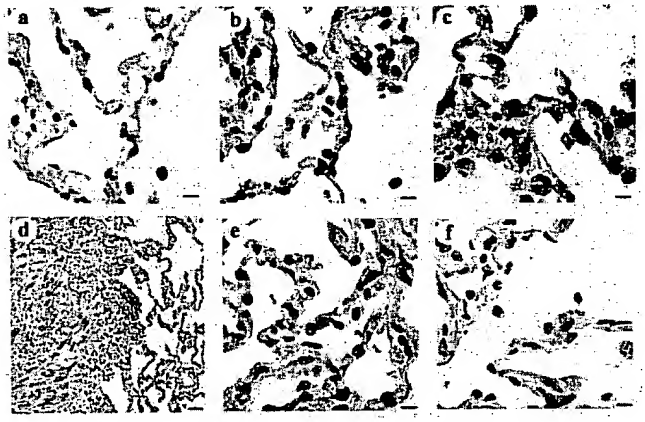



FIG. 2. ***In situ* hybridization analysis of *IL20RA*, *IL20RB*, and *IL22R* subunits in human lung.** Probes were detected using tyramide signal amplification signal amplification with Vector Red substrate, producing red positive signals. Probes used were *IL20RA* (*a*), *IL20RB* (*b*), and *IL22R* (*c*). Alpha actin-positive controls are shown in *d* at low magnification to show positive staining in smooth muscle fibers and in *e* at high magnification. A negative control probe is shown in *f*. *Bars* indicate 10 μm except *panel d*, where the *bar* indicates 100 μm.

STAT elements. In all cases where individual receptor subunits were transfected alone, there was no detectable luciferase production (data not shown). As described earlier (1), there was a dose-dependent increase in reporter luciferase activity in response to IL-20 treatment of BHK cells stably transfected with IL-20RA/IL-20RB (Fig. 3*A*). IL-19 and IL-24 were equipotent to IL-20 in this assay, with a half-maximal response between 20 and 40 pM. To confirm the specificity of activation, all three ligands were tested in the luciferase assay in the presence or absence of a heterodimeric IL-20RA/IL-20RB-soluble receptor (SR). A 50-fold excess of the SR resulted in nearly complete inhibition of IL-20 effects. Concurrent soluble receptor treatment resulted in a similar inhibition of IL-19- and IL-24-stimulated luciferase activity.

A similar set of experiments was performed using the same parental cell line stably transfected with IL-22R/IL-20RB and treated with increasing amounts of IL-19, IL-20, and IL-24 (Fig. 3*B*). IL-19 had no effect on this cell line. IL-20 and IL-24 were equipotent in stimulating luciferase output in this cell

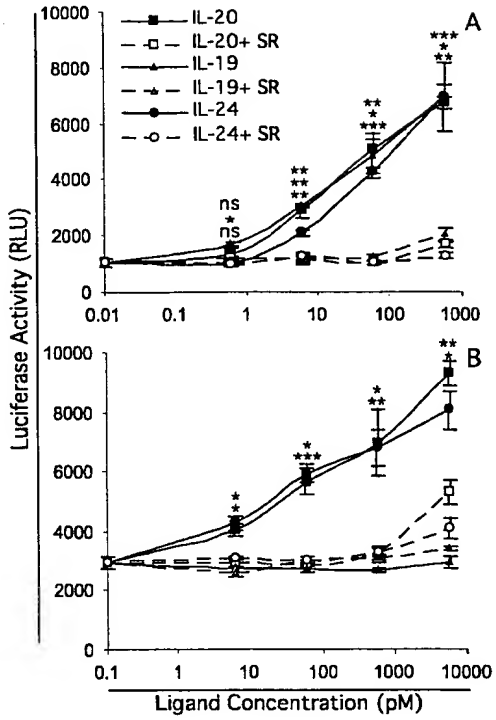



FIG. 3. **Luciferase reporter assays using BHK cell lines stably expressing (*A*) IL-20RA/IL-20RB or (*B*) IL-22R/IL-20RB.** The *solid symbols* and *lines* represent cytokine alone; *open symbols* and *dashed lines* represent cytokine with a constant 30 nM soluble heterodimeric IL-20RA/IL-20RB. Samples were compared with negative controls using a one-tailed *t* test; significance is indicated as: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. For *panel A*, significance is indicated for (*top* to *bottom*) IL-20, IL-19, and IL-24 at each point. For *panel B*, significance is indicated for (*top* to *bottom*) IL-20 and IL-24 at each point.

line, with half-maximal stimulation occurring around 60 pM. IL-20 and IL-24 activities were again blocked with SR treatment.

We next wanted to determine which soluble receptors were capable of blocking ligand activity. As described above, IL-20RA/IL-20RB heterodimeric soluble receptor blocked luciferase activity stimulated by IL-20, IL-19, and IL-24 on BHK cells bearing either combination of receptors. In similar luciferase assays, neither IL-20RA nor IL-22R was capable of blocking the activity of any of the three ligands (data not shown). In contrast, IL-20RB-soluble receptor alone did block the activity of IL-19 and IL-24, at >1000-fold excess for IL-19 and >100-fold excess for IL-24 (data not shown). Note that this soluble receptor is much less effective than the heterodimeric IL-20RA/IL-20RB, which fully blocked ligand activity at 50-fold excess (see above). Soluble IL-20RB had no effect on the activity of IL-20 at any concentration (data not shown). An additional binding assay (21), using soluble receptors to detect ligands transiently expressed in COS-7 cells, further confirmed the specific interaction of IL-20RB with both IL-19 and IL-24 (data not shown).

*Proliferation Assays*—We next evaluated proliferation of receptor-transfected BaF3 cells using Alamar Blue as an end point live cell number readout (Fig. 4). IL-20, IL-19, and IL-24 all stimulated proliferation of BaF3 cells stably transfected with both IL-20RA and IL-20RB (Fig. 4*A*). All three ligands showed equipotent activity on this cell line, with a half-maximal response occurring between 60 and 75 pM. BaF3 cells were also stably transfected with IL-22R and IL-20RB alone or in combination and treated with these ligands. Both IL-20 and IL-24 stimulate proliferation through IL-22R/IL-20RB (Fig. 4*B*), with a half-maximal response detected at ~6 pM: 10-fold

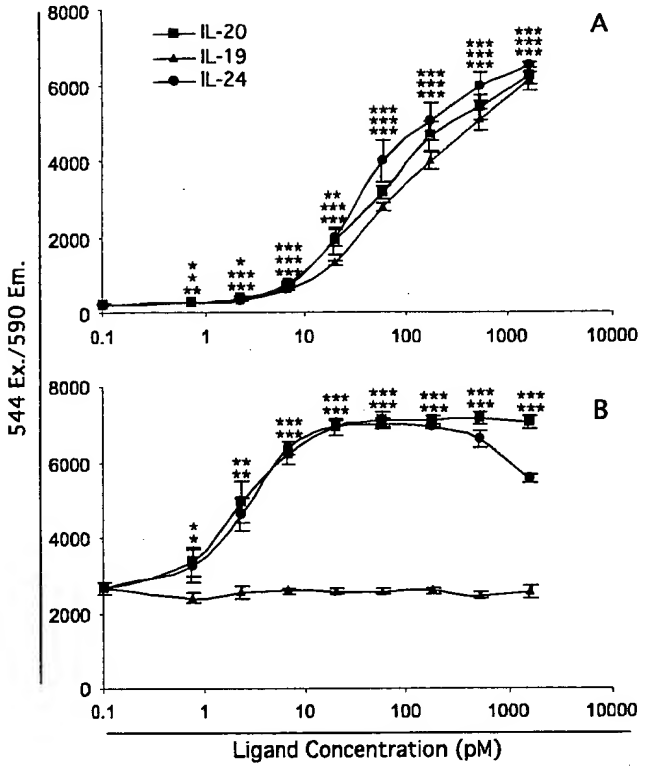



FIG. 4. **Proliferation assays using BaF3 cell lines stably expressing (*A*) IL-20RA/IL-20RB or (*B*) IL-22R/IL-20RB.** Cells were cultured for a total of 96 h in the presence of the indicated concentration of ligand. The fluorescent readout is proportional to the number of live cells in the culture well. Values plotted represent mean (±S.D.) for quadruplicate cultures. Statistical significance is indicated as in Fig. 3.

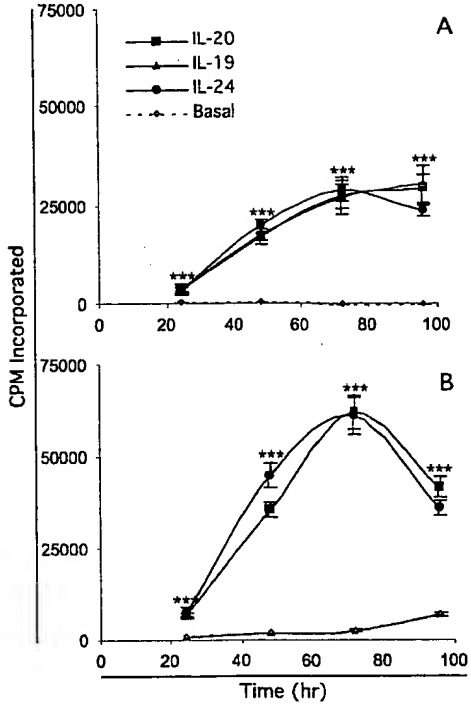



FIG. 5. **Tritiated thymidine incorporation assays using BaF3 cell lines stably expressing (*A*) IL-20RA/IL-20RB or (*B*) IL-22R/IL-20RB.** Cells were cultured for the times indicated in the presence of 60 pM of each ligand. Wells were pulsed 6 h prior to harvest. Values plotted represent mean (±S.D.) for quadruplicate cultures. In *panel B*, the plot representing IL-19 overlays the plot representing control (untreated) cells; in every other case the treated cells were highly significantly different ($p < 0.001$) from control.

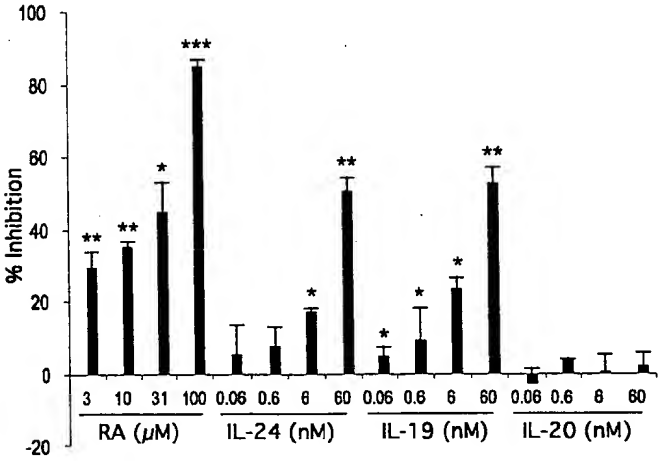



FIG. 6. **NIH:OVCAR-3 cells are growth-inhibited by IL-24 and IL-19.** Cells were treated for 48 h in the presence of the indicated concentration of each cytokine, or retinoic acid (*RA*) as a positive control. *Bars* represent mean (±S.D.) percent inhibition from triplicate cultures. Data were compared with untreated cultures using one-tailed *t* test; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

lower than that observed with BaF3 cells expressing IL-20RA/IL-20RB.

Given the differences in ligand potency on BaF3 cells expressing each receptor complex, we next evaluated the kinetics of the growth response in these cells using [$^3$H]thymidine incorporation assays. Fig. 5*A* shows that all three ligands stimulated a similar and continuous growth of BaF3 cells expressing IL-20RA/IL-20RB over a 72-h period. IL-20 and IL-24, but not IL-19, also stimulated the growth of BaF3 cells transfected with IL-22R/IL-20RB (Fig. 5*B*). The rate of growth stimulated by each ligand was similar for the same receptor complex, however, there were obvious differences in growth kinetics between the two cell lines. Cells transfected with IL-22R/IL-20RB proliferated much more rapidly, surpassing the maximal level of growth seen with the other complex within about 40 h. The decline in growth rate seen at 96 h is due to saturation of the culture. At its maximum, the growth rate of these cells is about double that of the cells expressing IL-20RA/IL-20RB.

*Growth Inhibition Assays*—To perform assays on a cell type endogenously expressing all three receptors, we performed RT-PCR analysis on a variety of cell lines and identified one, NIH:OVCAR-3 (20), which expresses *IL20RA*, *IL20RB*, and *IL22R* (data not shown). Because IL-24 is known to inhibit growth of a variety of tumor cell lines (3, 15), we chose to measure ligand-induced growth inhibition in these cells. IL-19 and IL-24, but not IL-20, treatment resulted in a dose-dependent growth-inhibitory effect (Fig. 6), with half-maximal responses at about 30 nM: 500- to 5000-fold greater than those measured for proliferation or reporter activation in the receptor-transfected BaF3 and BHK cell lines. This result was confirmed in three independent assays. Concurrent STAT reporter activation experiments with the NIH:OVCAR-3 cells revealed that treatment with IL-19, IL-20, or IL-24 did not result in activation of the STAT pathway (data not shown). Similar results were obtained when a nuclear translocation assay was performed.

*STAT Translocation Assays*—To determine whether STAT recruitment changes with increasing ligand doses, we directly analyzed STAT protein nuclear translocation using the BHK cell lines stably expressing each receptor complex. These assays measure activation of individual signaling elements by measuring their localization in the nuclear *versus* cytoplasmic compartment in resting or stimulated cells (22). For our assays, STAT1- and STAT3-specific detection reagents were used. In

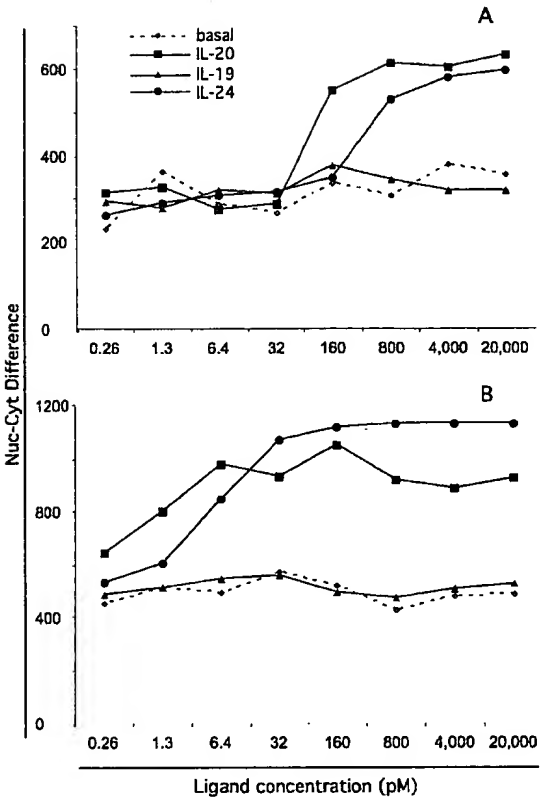
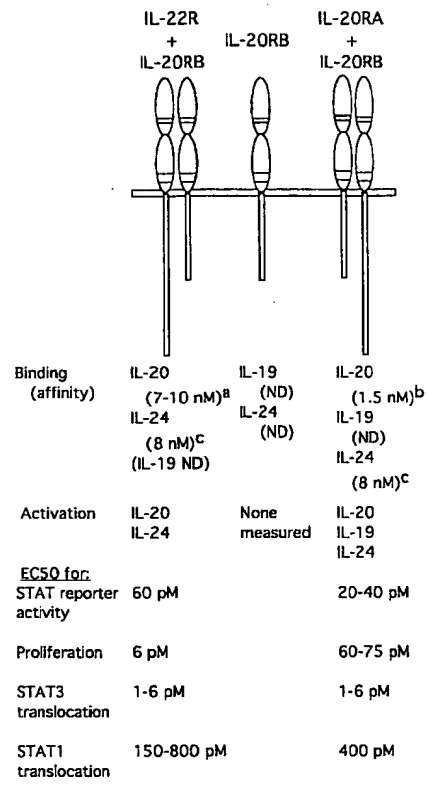



FIG. 7. **STAT translocation assays using BHK cells expressing IL-22R/IL-20RB.** Each data point represents the mean difference between nuclear and cytoplasmic localization of STAT1 (*A*) or STAT3 (*B*) for each treatment; at least 100 individual cells were analyzed for each point. Statistical significance markers and *error bars* were omitted for clarity; due to the large number of events measured, differences as small as 10% are highly significant.




| | IL-22R + IL-20RB | IL-20RB | IL-20RA + IL-20RB |
|---|---|---|---|
| Binding (affinity) | IL-20 (7-10 nM)[a]<br>IL-24 (8 nM)[c] (IL-19 ND) | IL-19 (ND)<br>IL-24 (ND) | IL-20 (1.5 nM)[b]<br>IL-19 (ND)<br>IL-24 (8 nM)[c] |
| Activation | IL-20<br>IL-24 | None measured | IL-20<br>IL-19<br>IL-24 |
| EC50 for: | | | |
| STAT reporter activity | 60 pM | | 20-40 pM |
| Proliferation | 6 pM | | 60-75 pM |
| STAT3 translocation | 1-6 pM | | 1-6 pM |
| STAT1 translocation | 150-800 pM | | 400 pM |

FIG. 8. **Summary of the activities of each ligand on each receptor complex.** Affinity values are from (*a*) our preliminary estimate (data not shown); (*b*) Ref. 1; (*c*) Ref. 14.

BHK cells bearing the IL-22R/IL-20RB receptor complex, both IL-20 and IL-24 robustly translocate STAT1 and STAT3 (Fig. 7, *A* and *B*). The STAT3 translocation reaches the half-maximal level at 1–5 pM cytokine, whereas the STAT1 translocation is half-maximal at 150–800 pM and has dropped to background levels at ligand concentrations below 30 pM.

We previously reported nuclear translocation of STAT3 but not STAT1 in response to IL-20 (1); similarly, both IL-19 and IL-24 translocate STAT3 in BHK cells bearing IL-20RA/IL-20RB (data not shown). In this set of experiments, we did observe some STAT1 recruitment at high IL-20 doses. Similar results were obtained with IL-19 or IL-24, with half-maximal STAT1 translocation at about 400 pM (data not shown).

## DISCUSSION

We show that functional differences exist in the IL-20 subfamily in which IL-20, IL-19, and IL-24 exhibit sharing of receptor complexes. Although STAT reporter activation and preferential activation of STAT3 *versus* STAT1 are similar between complexes, differences become apparent when ligand-induced proliferation is compared between receptor complexes. An ovarian carcinoma cell line endogenously expressing all three receptor subunits also responded differentially to the ligands; IL-19 and IL-24 were growth inhibitory, whereas IL-20 was not.

Our expression data show that, in most tissues expressing the common subunit *IL20RB*, the only other subunit expressed is *IL20RA*. Thus in the majority of cases the cytokines in this subfamily will be expected to signal through IL-20RA/IL-20RB. *IL22R* is found in a few tissues lacking *IL20RB* expression; these include adult and fetal liver, colon, small intestine, and pancreas. In these tissues its role as part of the IL-22 receptor is expected to predominate, consistent with reports of IL-22 proinflammatory activity on hepatocytes (5) as well as recent data showing IL-22 activity on pancreatic acinar cells (23). Interestingly, we did not find any tissue or cell type in which *IL22R* was expressed with *IL20RB* in the absence of *IL20RA*; rather, tissues co-expressing *IL22R* with *IL20RB* also expressed *IL20RA*. We detected *IL20RA* and *IL22R* messages in lung immune infiltrates, which consist largely of specialized macrophages (24, 25). These messages are detectable neither in peripheral blood leukocytes (Fig. 1 and Table I) nor in resting or activated peripheral immune subsets (26). Thus they appear to be up-regulated in immune cells only under certain maturation or stimulation conditions. Taken together, these data suggest that, although the primary receptor complex in most cell types is IL-20RA/IL-20RB, in some cell types expressing all three receptor subunits the net signal transduced by a given ligand would depend upon a more complicated set of interactions between that ligand and the two signaling complexes.

We studied the types of signals transduced by each receptor complex separately in transfected BHK and BaF3 cells. Although the $EC_{50}$ values for STAT reporter activation in BHK cells were very similar for all ligand/receptor combinations, there were substantial differences between receptor complexes in their ability to promote proliferation of BaF3 cells. Both IL-20 and IL-24 exhibit 10-fold lower $EC_{50}$ values for proliferation of BaF3 cells bearing IL-22R/IL-20RB as compared with cells bearing IL-20RA/IL-20RB (summarized in Fig. 8). Although it could be argued that this result may be due to higher ligand affinity for IL-22R/IL-20RB or to higher expression levels of that receptor complex on the assay cell line, it should be noted that the $EC_{50}$ values for both proliferation and reporter activation are 25- to 200-fold below the measured affinities of the ligands for receptors, where these values are known (Fig. 8). This suggests that these signaling events require only

fractional receptor occupancy, and are thus reasonably insensitive to small differences in affinity or receptor number. A more likely explanation is that the signal transduced by IL-22R/IL-20RB differs from that of IL-20RA/IL-20RB in a way that favors proliferation.

To further study the differences in signal transduction, we examined nuclear translocation of individual STAT proteins in transfected BHK cells. IL-20 and IL-24 appear equivalent in function when acting through IL-22R/IL-20RB, with robust activation of STAT3 showing $EC_{50}$ values of 1–5 pM. The $EC_{50}$ value for STAT1 activation is substantially higher, in the 150–800 pM range. When the STAT translocation assays were done on BHK cells bearing IL-20RA/IL-20RB, all three ligands activated STAT3 equivalently. Activation of STAT1 through this complex was slightly more robust with IL-19 and IL-24 as compared with IL-20, but the $EC_{50}$ values for both activities were consistent with those observed for cells bearing IL-22R/IL-20RB. Taken together our STAT activation data show that at low (below 100 pM) ligand concentrations, STAT3 activation is favored over STAT1 activation through either receptor complex.

STAT3 is involved with signal transduction through a wide variety of receptors, and, not surprisingly, ablation of the STAT3 gene results in embryonic lethality (for review, see Refs. 10 and 27). As a result, the critical actions of STAT3 are studied in tissue-specific knockouts. Because the three receptor subunits we studied are all expressed in skin, we were particularly interested in the recent report of keratinocyte-specific ablation of STAT3 (28). Keratinocytes from these mice failed to migrate in response to epidermal growth factor, transforming growth factor $\alpha$, hepatocyte growth factor, or IL-6, and this defect resulted in impaired wound healing and abnormal hair cycles. The keratinocytes showed normal proliferative responses, leading the authors to conclude that the proliferative response was due to activation of one or more STAT3-independent pathways, whereas the migratory response was STAT3-dependent.

We have shown that IL-19, IL-20, and IL-24 activate STAT3 through either receptor complex. We previously reported that IL-20 also activates STAT3 in the human keratinocyte cell line HaCaT and have shown marked synergy of IL-20 with epidermal growth factor, IL-1$\beta$, or tumor necrosis factor $\alpha$ in STAT-luciferase reporter assays (1). Our IL-20 transgenic phenotype and our observation of high receptor expression in psoriatic skin (1) would appear to favor a role of IL-20 in proliferation of keratinocytes. We did not observe changes in keratinocyte migration in IL-20 transgenic mice, but it is possible that such a defect might have become apparent had the mice survived beyond the neonatal period. Interestingly, transgenic mice overexpressing IL-19 have no apparent skin phenotype,[3] and we are unaware of any changes in skin caused by administration of IL-24 (although IL-24 is up-regulated in wound healing (29)). One hypothesis is that the hyperproliferation of keratinocytes seen in IL-20 transgenics is due to activation of a STAT3-independent pathway unique to IL-20. Future work with mice lacking each of the individual receptor subunits will prove helpful in determining the relative importance of each ligand in skin structure and remodeling.

Growth inhibition assays on NIH:OVCAR-3 cells revealed a functional divergence among the three ligands. IL-19 and IL-24 at doses above 600 pM inhibited the growth of this cell line, whereas IL-20 had no effect. To our knowledge, this is the first report of cytostatic effects of IL-19. The growth inhibitory effect that we detected on OVCAR cells did not appear to be cytotoxicity, because the cells grew normally following removal of the cytokines. The cytostatic effect does not seem to be a general response to treatment with these ligands, because none of the ligands at any concentration affects the growth rate of BHK cells transfected with either receptor complex (data not shown). Several groups have tried to dissect the putative pathways involved in the growth inhibitory activity of IL-24 using adenoviral delivery of this protein. The early work described this activity of IL-24 as growth-suppressing (3). Su *et al.* (15) showed nucleosomal DNA degradation in human breast cancer cells infected with adenoviral IL-24. These previous studies were done using adenoviral delivery of IL-24, whereas our experiments utilized purified proteins. Future experiments will need to better differentiate which effects of IL-24 are specific only to adenoviral delivery of this protein.

Because the growth inhibitory effect does not correlate with the receptor complex specificities of the ligands, it may be that the growth inhibition associated with IL-19 and IL-24 results from utilization of another signaling pathway. This pathway could involve signaling through the double-stranded RNA-dependent protein kinase, as has recently been observed in lung cancer cell lines (30). Use of an alternative pathway is supported by the failure of any of the ligands to activate the STAT/luciferase reporter or nuclear translocation assay in this cell line. Another possibility is that aberrant receptor complexes form at saturating doses of cytokine. Because IL-19 and IL-24 both bind to IL-20RB alone, a quality that IL-20 lacks, they could induce homodimerization of IL-20RB. They could also induce the formation of heterodimers between IL-20RB and an unknown additional receptor subunit. A third possibility is that IL-20 has a lower affinity for the receptor complexes than do IL-19 and IL-24, such that saturation kinetics are reached at the concentrations tested for the latter two but not for IL-20. An example from the interferon $\alpha/\beta$ system supports this hypothesis. A low affinity ligand, interferon $\tau$, has antiviral properties similar to those of interferon $\alpha$ but is at least 30-fold less toxic. Thus in this system toxicity is associated with saturation binding and is related to the $K_d$ value, whereas maximal antiviral activities are induced with only fractional receptor occupancy (31). A similar mechanism could be responsible for the disparate cytostatic effects of IL-19 and IL-24 as compared with IL-20 despite their similar activities in every other type of assay.

We have demonstrated substantial functional differences between the two receptor complexes in the IL-20 subfamily, reflected in proliferation assays. The growth inhibition induced by IL-19 and IL-24 in the OVCAR cells revealed a novel difference between ligands that is not explained by receptor specificity. Future work will be needed to dissect this ligand/receptor system further to better explain the biological differences seen in this family.

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